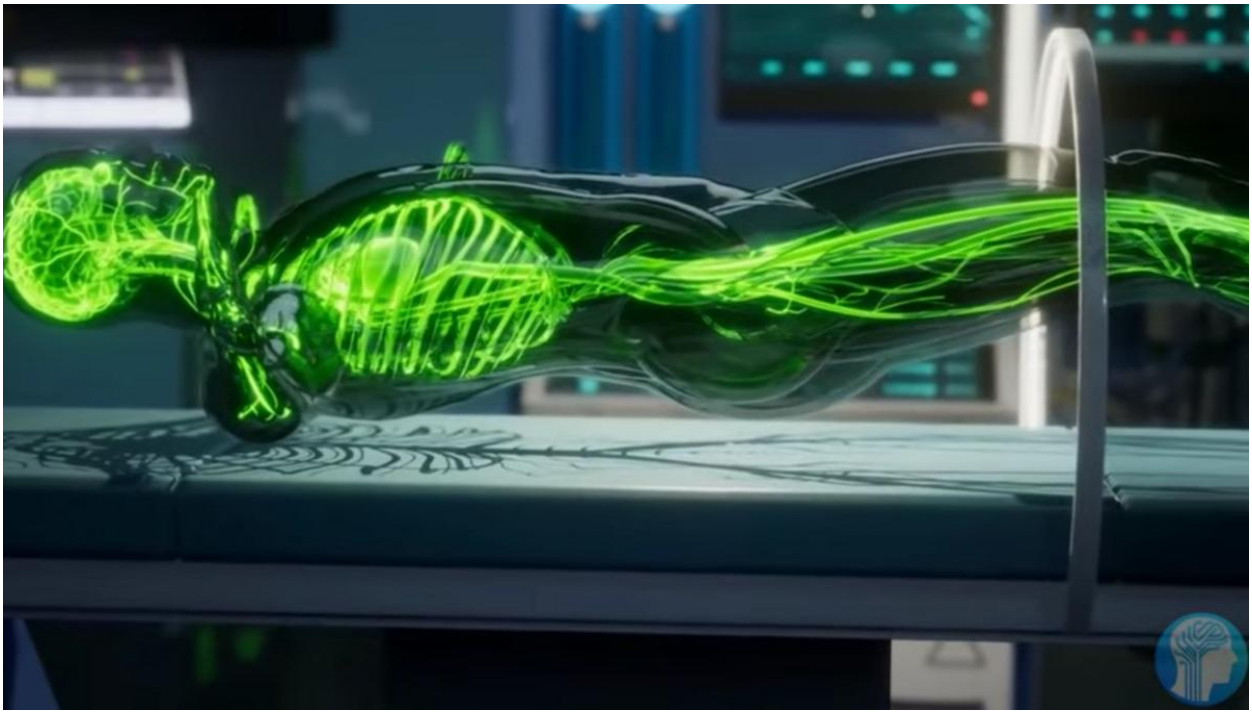
MEDICAL CELLULAR REJUVENATION TECHNOLOGY BED (Ongoing Research)

From the sci-fi novel, ‘OffWorld Man Anthology: The Ultimate Collection’

Find the REAL TECHNOLOGY Precursors



-THE REJUVENATION BED TECHNOLOGY WILL INVOLVE CELL BIOLOGY

-DEVELOPMENT OF NON-INVASIVE WAYS TO DIAGNOSE, AND DELIVER NUTRITION TO EACH INDIVIDUAL HUMAN CELL, OF 37 TRILLION CELLS.

-NON-INVASIVE; TACHYONS? RED LIGHT THERAPY MUST ALSO BE USED IN COMBINATION. COLD LASER/ PHOTON/ TACHYON DELIVERY SYSTEM?

-THERAPY MUST NON-INVASIVELY PENETRATE COMPLETELY THROUGH THE BODY, THROUGH BONE, TO THE MARROW. MRI MACHINE-LIKE.

-THINK PAST THE IMAGINARY, IDEATED SCI-FI ASPECT AND FIND THE REAL-WORLD PRECURSORS TO THE TECH SOLUTION.

AI AGENT RECOMMENDS BIOELECTRONICS, BIOINFORMATICS, AND POSSIBLY CRYOGENICS OR LOWERING THE BODY TEMPERATURE TO SLOW THE METABOLISM.

IT WILL LOOK SIMILAR TO AN MRI MACHINE WITH VARIED CANISTERS OF NUTRACEUTICALS OR NOOTROPICS ATOP IT USING OSMOSIS TO FEED THE UNIT DURING TREATMENT. Since the human body is composed of approximately 37 trillion cells that contain roughly 85% water, this makes osmosis a very important concept.

Tachyon, because it is the source particle of energy that organizes and energizes the SOEFs, provides the potential to restore harmonious healing frequencies. Creating a neg-

entropy effect, Tachyon organizes that which is disorganized.

FIND THE PRECURSORS TO THE FOLLOWING FUTURE-TECH

Speculative Future Technology: **Nootropic or nutraceutical cold laser anodyne human cell rejuvenation treatment bed**

A mitochondrial cellular rejuvenation and telomere restoration procedure starts with the patient lying on an air gel glide pad. Two wide, flat counter-rotating rings configured on a sliding apparatus under his resting body cycled to the completion of a three-hour treatment session. A combination of mild pulses of low-level sapphire crystal refracted laser and infrared light, emitted from hundreds of pinpoints, millimeter-sized pores on the inner surface of the spinning rings. The light saturated the mitochondria of every cell in his body with a rejuvenating disc-shaped collective wall of healing micronized nootropics via violet-blue light energy and pulsed magnetic prime resonances. The apparatus glided slowly one-half inch at a time, making barely a beep at four-second intervals from the crown of the head to the soles of his feet repeatedly, ensuring that every square centimeter of the body received equal amounts of nano-particle-infused nourishing light. The painless procedure feels like a massage. The porous ring apparatus delivers cellular nanoparticlized nootropics or nutraceuticals via Edser photonic quanta or tachyon energy that calibrates and rejuvenates the mitochondria of the cell with no damage.

The human body is an aggregate of numerous cells, which continuously grow, develop, split, regenerate, and die. By splitting up, cells renew themselves. For adults, roughly 25 million cells are splitting every second, and blood cells are constantly renewing at a rate of about 100 million per minute.

In the process of cellular split-up and renewal, the charged bodies of the nucleus and extranuclear electrons as the basis of a unit cell are moving and changing ceaselessly at a high speed as well, emitting electromagnetic waves without interruption. The signals of electromagnetic waves emitted by human bodies represent specific conditions of health, good or bad health and diseases, etc. The

conditions of life via electromagnetic wave signals can be analyzed. The quantum resonant magnetic analyzer function of the deep penetrating, pulsed sapphire crystal refracted low-level laser and infrared light-based looping bio-therapy system determines what nutrients each cell is deficient in.

Weak magnetic frequency and the energy of the body's cells are collected, analyzed, and compared with the standard quantum resonant spectrum of nutrition, disease, and other indicators incorporated into the rejuvenation unit to judge whether the sample waveforms are irregular using the Fourier approach calculations. Analysis and proper nutraceutical treatment can thus be applied to the main problems based on the result of waveform analysis, as well as standard curative proposals.

The rejuvenation bed is a rapid, non-evasive spectral testing and treatment method for curative effects medicine and checking of sub-health conditions. The main analysis checks include cardiovascular and cerebrovascular conditions, bone mineral density, blood lead, rheumatism, lung and respiratory tract, nephropathy, stomach and intestines, liver and gall, cranial nerves, gynecology, prostate, cancer disease, and trace elements of vitamins and minerals.

The machine utilizes a quantum resonant magnetic analyzer and a deep penetrating, pulsed sapphire crystal refracted low level laser and infrared light based bio-therapy system, much like photosynthesis, to deliver micronized nootropics or nutraceuticals, antioxidants, and essential monoatomic elements such as: Astragalus,nanoparticle Gold, nanoparticle Silver, nanoparticle Platinum, nanoparticle Copper, Organic Sulfur, Mega Hydrate, Nascent Iodine, and phyto-nutrient dense nanoparticlized micro compounds such as R-Lipoic Acid, Carnosine, Polyphenols, Green Tea extract, Curcumin extract, Resveratrol, Bio-Flavonoids, Pine Bark extract, Acetyl-L-Carnitine Arginate DiHCI, Benfotiamine, Pyridoxal 5-Phosphate, PQQ, Luteolin, Pterostilbene, Fistetin, Resveratrol, Tyrosyl, Hydroxytyrosol, DHEA, CoQ10, Shilajit, 25-Hydroxy D, EPA or Ecosapentaenoic Acid, C, E, B6 and B12 complex, K complex, Aquaglycerolporins-3, Glycosaminoglycans, Chlorogenic acid, Magnesium-L-threonate, Lutein, Gingko, Chlorophyllin, Lycopene, Gamma Tocopherol, Curcumin, Reishi extract, Ellagitans, Anthocyanins, Pignoginol, Carotenoid, Astaxanthin, Polypodium Leucotomies extract, S-Adenosyl-Methionine, Creatine, Coffee berry extract, Fruit XB, Hops extract, Sinoprene, Kelated Boron, AKBA

Boswellian extract, Sirtuin enzymes 1-7, Human Telomerase enzyme, BubR1 protein and Ketose; to slowly repair and boost critical cellular function.

The addition of sapphire crystal refraction allowed for a greater increase in the utilization of both laser and infrared light by the human organism. The system uses molecular scanners and an advanced algorithm similar to the DNA CRISPR CAS 9 high-end computer, sequencing the 6 billion letters of the human genetic code, the recorded history of human genetic evolution, to diagnose, and predict future diseases, and heal the human body's 36+ trillion individual aging acetyl ethyl phosphate cells through deep-penetrating light inducted rejuvenation. Every organic species on the planet is a DNA-based bio-decoding and converting software system.

The sapphire crystal laser and infrared light biotherapy also deliver negative ions to the body through the skin's surface, which energizes the body for optimal health. When negative ions are applied to the body, the calcium and sodium circulating in the blood are ionized. This increases the blood's pH and has an overall alkalizing effect on the body. Proper blood pH is essential for optimal health. Negative ions applied to the body help regulate blood pH. When the number of negative ions in the blood increases, there is an increase in inter-cellular communication. Cellular metabolism is facilitated, and as a result, there is an increase in cellular nutrient uptake and cellular hydration. In addition, cellular waste materials are efficiently excreted. With the increase in cellular metabolism, optimal cell function is rapidly revived.

Far infrared rays penetrate six to eight inches into the human body. These rays not only benefit the skin and muscle tissue on the surface of the body but penetrate deeper into tissues. The far infrared penetrates into the lymph glands, blood vessels, nerves, and key organs. The rays optimize the health of these tissues in a number of ways. Among the waves within the energy spectrum coming from the Sun, far infrared waves are by far the safest and most beneficial electromagnetic energy available for the human body, directly affecting all metabolic and cellular functions.

The sapphire crystal laser and infrared light biotherapy improves circulation and cardiovascular function; the light waves raise body temperature, warming the

blood and expanding the blood vessels. There is an increase in peripheral blood flow and volume, improving circulation and heart function. The unit improves the function of the body's immune system and combined with the detoxification of harmful toxins and waste products, one's overall health and resistance to disease are greatly improved.

The intra-cavity light passes through the cells as the rings travel from the crown chakra to the heels. There is a combination of deep tissue 7.5-to-7.83-megahertz magnetic harmonic prime resonance, far infrared, and photonic tachyon quanta stimulation. The machine hums, making “OHM” and “HU” sounds, like Tibetan monks in unison, tuning the body right down to the DNA to its synchronized harmonic waves. The prime resonance spectrum is quite powerful and works well with the Edser photonic therapy, which also activates the lymphatic system to create hyaluronic acid, the so-called Youth Molecule, connecting the structures of the cell.

The Edser LIGHT-BASED healing therapy also increases the pineal gland’s production of natural DMT, the so-called transcendental spirit molecule. My treatment also involves filtering the biome for cancers and cancerous genes such as P-53, RANi interference and somatic gene therapy to alter longevity and reverse the aging process. Treatments can be infinitely tailored to the patient’s diagnosis.

Treatment also activates telomere gene enzymes that maintain the ends of the cell chromosomes. This machine helps the body to create more telomerase and slows down significantly the baseline rate at which the telomeres shorten each time a cell divides.

When telomeres get too short each time a cell divides, the cell can no longer make copies of itself and the tissue and organ systems that rely on continued cell replication, namely the 200 different types of tissues in the human body, begin to falter.

The cell mitochondria convert glucose into energy. As the mitochondria age, they spew out increasing amounts of free radicals that hamper energy production and damage the entire cell, accelerating the body’s all systems decline, the classic aging process. Tissues and organ systems that depend on cell division have a limited amount of reserve capacity.

The cells that play the greatest role in the body's decline, the neurons and the heart muscle cells hardly replicate. Heart health depends heavily on the endothelial cells, and brain health relies on the glial and Schwann cells. This machine can rejuvenate these cells and any of the cells in an organic system.

The healing unit relieves pain and helps peripheral blood vessels dilate, bringing relief and healing to muscles and soft tissue injuries. Increased blood circulation carries off metabolic waste products and delivers oxygen-rich blood to oxygen-depleted muscles, so they recover faster. As one relaxes in the gentle heat of the far infrared, one's body is actually hard at work, producing sweat, pumping blood, and burning calories. A single session burns as many calories as rowing or jogging for 30 minutes. The healing therapy eases joint pain and stiffness from many kinds of arthritic and muscular-skeletal disorders. The healing unit was effective in the treatment of sprains, neuralgia, bursitis, muscle spasms, joint stiffness, and many other muscular-skeletal ailments. Stiffness, aches, and soreness that come with aging are reduced and eventually eliminated. The healing unit reduces stress and fatigue. The gentle warmth of the far infrared rays helps to soothe nerves and relaxes tight or knotted muscles. The end result is reduced stress and improved energy.

The sapphire crystal laser and far infrared light biotherapy produce negative ions that penetrate the skin deeply, helping detoxify impurities from the skin and lymphatic system. In addition, exfoliation is greatly enhanced by the far infrared, rapidly removing dead skin cells. Increased circulation draws the skin's natural nutrients to the surface, rejuvenating the skin's health and appearance. The healing sessions remove bodily toxins and assist in detoxification. Far infrared helps to detoxify the body in several important ways. Increased blood circulation stimulates the sweat glands, releasing built-up toxins and waste. Daily sweating can help detoxify the body as it rids itself of an accumulation of potentially carcinogenic heavy metals, alcohol, nicotine, sodium, sulfuric acid, cholesterol, and uric acid. In addition to causing the body to sweat, far infrared is capable of removing toxins via several other bodily systems.

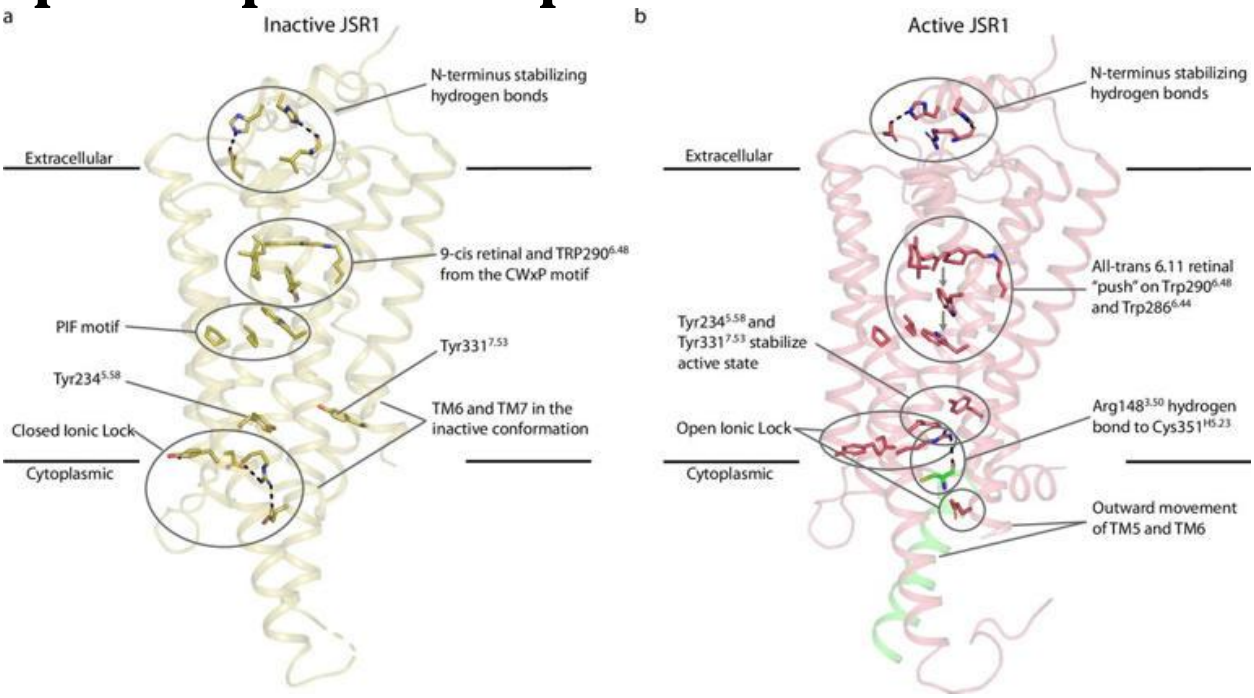
The healing unit is controlled by advanced A.I. computer technology that monitors all aspects of its performance by sensors distributed throughout the

resting air gel pad. Reverse-engineer this technology and democratize it for the benefit of all humanity, not just some-- goddamned breakaway civilization.

FIND THE PRECURSORS TO THE FUTURE TECH

RESULTS SO FAR AS OF 2025

On the way to light-controlled medicine: Researchers elucidate the structure of specific photoreceptors



Activation of JSR1 by the agonist all-trans 6.11 retinal. Credit: Nature Communications (2024). DOI: 10.1038/s41467-024-53208-2

Researchers in biology and medicine have long dreamed of controlling the activities of cells without, for example, having to use chemicals. After all, in a structure as complex as an entire organism, unwanted side-effects can often arise.

The ideal solution would therefore be a type of remote control for cells, which would allow the functions of individual organs to be better examined and understood, and could even be used for therapeutic purposes. Remote control using light would be ideal for this, as it would enable organs and tissues deep inside the body to be influenced in a very selective and non-invasive way.

However, such a process also requires a cellular light receiver in the relevant organs. The receptors that receive light impulses in the retina of our eyes—called rhodopsins—could be suitable for this. With such photoreceptors, it might be possible to switch certain cell functions on and off using a light impulse.

This would work more rapidly and in a more targeted manner than drugs, which take a long time to take effect and often have unwanted side-effects because they cannot simply be activated in just one specific organ.

In the neurosciences, something similar is already working and is currently being tested in animal models to investigate brain diseases such as Parkinson's and epilepsy: Light-controlled ion channels from single-celled organisms are being incorporated into neurons using genetic engineering.

In the animal model, these ion channels in the cell membrane open when exposed to blue light, for example, and allow positively charged ions to flow into the neuron. In a chain reaction, further channels open, creating an electrical signal—the neuron becomes active.

A new kind of optogenetics

But such light-controlled ion channels only work in nerve cells. The goal of this research, however, is to stimulate other cells and organs in the organism to control a variety of bodily functions. For example, one could investigate the heart's natural pacemaker, or the mechanisms of chronic pain, anxiety, depression, and other mental illnesses.

It might be possible to develop effective cell therapies for hormonal malfunctions as well as immune, heart, and other diseases, including cancer.

To this end, researchers led by Gebhard Schertler of the PSI Center for Life Sciences are working on a new kind of optogenetics. In this approach, it is light

receptors similar to the rhodopsins in our retina that become active: Triggered by a light pulse, they couple to proteins in the cell and thus initiate specific cellular signaling processes that take place in all organs.

The PSI researchers have joined forces with leading colleagues in Germany and England. Their project, Switchable rhodOpsins in Life Sciences (SOL), has three goals:

1. Find rhodopsins that can do this and elucidate their structure to better understand how they work.
2. Modify such rhodopsins, using molecular biological methods to optimize them for switching processes in various bodily functions.
3. Use the switches to better understand the signaling mechanisms of the proteins; use them as a tool in research and, on that basis, develop gene therapeutics.

The structural elucidation of proteins is a core competence of PSI, thanks to its high-resolution large research facilities. And PSI researchers have now made two significant steps towards SOL's first goal, as they report in two new studies.

First, they succeeded in finding a suitable rhodopsin and modifying it in such a way that it remains stable in the active state and thus can be examined. And second, the structure of this active state was clarified using a cryo-electron microscope at ETH Zurich.

A switch that bends and stretches

Rhodopsins are proteins. They are among the most important photoreceptors in the animal world. They have an elongated molecule in the middle, called retinal, that is derived from vitamin A. When a light pulse hits this molecule, it absorbs the energy and changes its shape within a quadrillionth of a second. A curved molecule—called the 11-cis form—becomes an elongated one—called the all-trans form. Through this transformation, the retinal also changes the structure of the entire rhodopsin so that it can now bind to other proteins in the cell membrane, so-called G proteins.

Therefore these light-sensitive rhodopsins also belong to the GPCRs (G protein-coupled receptor) family, as rhodopsin-G protein complexes stimulate other

proteins to react, triggering a whole series of biochemical processes leading, for example, to the transmission of a visual signal to the brain.

The human body possesses hundreds of different types of GPCRs, which are located in the cell membranes, receive signals from the outside, and pass them along to the inside of the cell. In this way, they control many bodily functions. That's why roughly 40% of all medications target GPCRs with active ingredients that dock onto their receptors.

The advantage of simple photoreceptors

Rhodopsins are found in the retina of the human eye. In the rod cells, for example, they are responsible for distinguishing between light and dark at night. However, like those of most vertebrates, these rhodopsins are monostable.

This means that once the retinal is changed by light, it leaves the protein and has to be regenerated. Only then is it available for the next switching process. This is too complicated to allow this molecule to be used effectively as an optogenetic switch, since enzymes would also have to be used to regenerate it.

Many invertebrates, such as squid, insects, and spiders, have bistable rhodopsins. "From an evolutionary perspective, these are actually a more primordial form of rhodopsins, and less sensitive," says Gebhard Schertler. They offer advantages for optogenetics, however: The retinal remains in the protein after being switched on, and with a second light pulse it can immediately return to its original form and switch the cellular process off again.

The rhodopsin of a jumping spider species, for example, proved to be robust and easy to produce, unlike other bistable rhodopsins. This qualified it as a possible optogenetic switch.

With the Swiss Light Source SLS at PSI, it was possible to determine the molecular structure of this spider rhodopsin in its inactive ground state. But before it could be used as an optogenetic switch, its structure in the active form also had to be known precisely. This state, however, when the retinal is stretched and the rhodopsin binds to the G protein, is extremely short-lived.

How to make proteins happy

In one study, which was recently published in the *Proceedings of the National Academy of Sciences*, lead author Matthew Rodrigues now reports how they managed to stabilize the active state to be able to elucidate its structure: by making a tiny modification to the retinal.

"The properties of the retinal remain the same, but the modification—one small additional molecular ring—ensures that it apparently fits better into the binding pocket of the protein," reports Rodrigues. "It stays there for hours. As we structural biologists say, it's happy." Now the conditions were in place to examine the structure of the active rhodopsin in conjunction with a G protein.

A mixed protein

In a second study, now published in *Nature Communications*, first author Oliver Tejero and last author Ching-Ju Tsai did exactly that. "However, as expected, it was found that a spider protein (rhodopsin) naturally never fits optimally with a human protein (the G protein)," says Tsai. "So we compared spider G proteins with those of humans and assembled a chimera from both forms."

The researchers replaced the end part of the gene sequence of the human protein, which contains the code for the docking site, with that of the spider.

With additional genetic modifications in the actual light receptor, they addressed another problem: The spider rhodopsins are both activated and deactivated by light of the same wavelength.

"This means that a light pulse produces a hopeless hodgepodge of activated and deactivated states in a cell sample," says Tsai. Naturally, this is bad for a switch that is intended to turn on or off in a targeted manner. "With our modifications, we have ensured that switching on and off now takes place with different colors of light."

However, such "color tuning" by means of genetic engineering is only just beginning. The next step in the fundamental research into these new optogenetic

switches will now be to find out how the proteins involved need to be designed to enable control using other colors of light.

This would then make it possible to selectively switch different cell functions on or off. It is also a matter of constructing the switches so that they are not only sensitive to blue, orange, and green light, but also, for example, to infrared light.

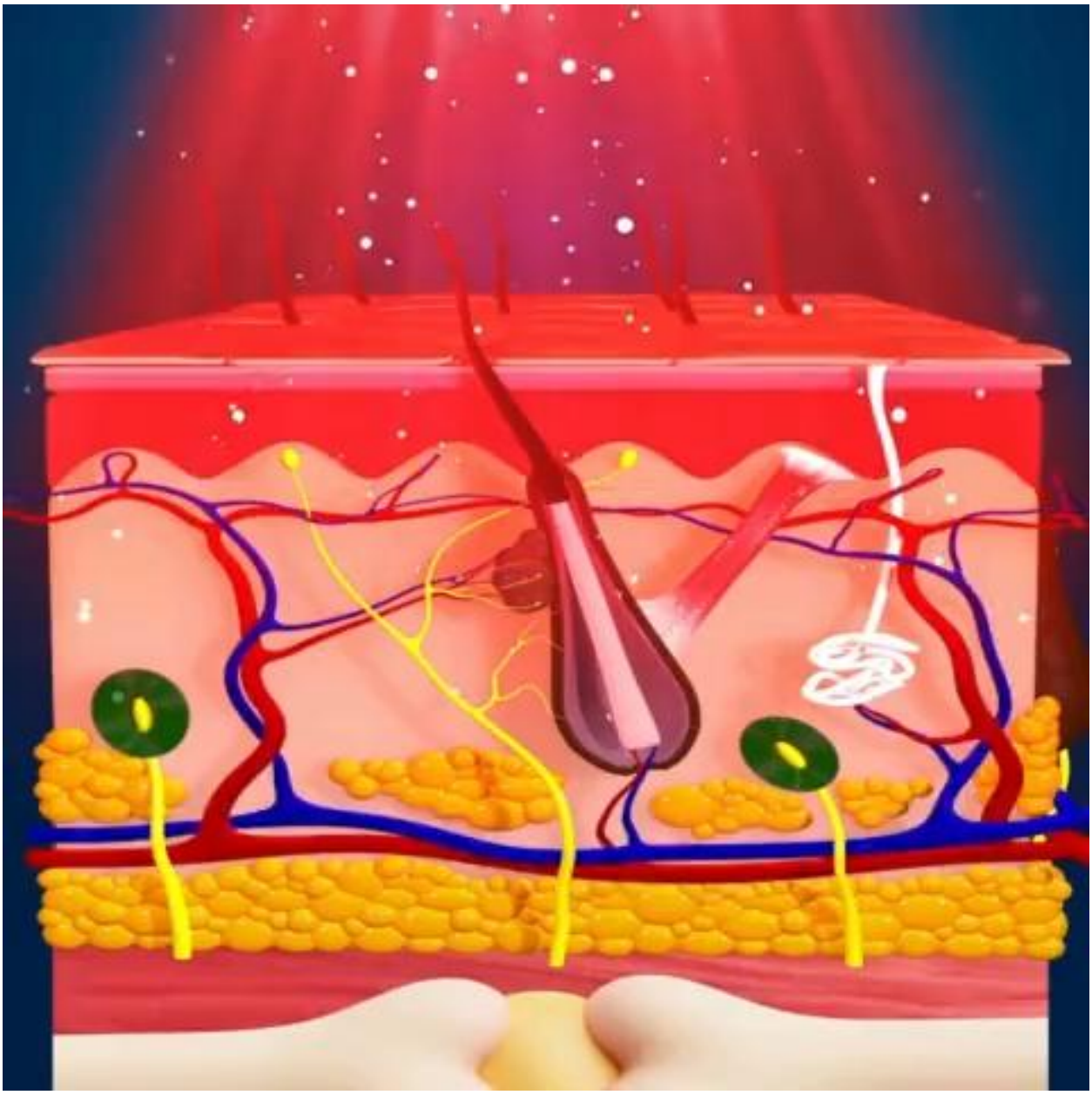
"The big question remains, if optogenetics is actually to be used in everyday medical practice, how the light will get to the rhodopsin," says Rodrigues. "You could implant the light source into the body. But the much more elegant and gentler method would be to work with infrared light. This can penetrate body tissue."

The largest part of the protein engineering, project leader Schertler confirms, is still to come, now that the structural basics are known. Ultimately, the goal is to put together a whole assembly kit of light-activated GPCRs that can be used for various purposes in the organism.

More information: Matthew J. Rodrigues et al, Activating an invertebrate bistable opsin with the all-trans 6.11 retinal analog, *Proceedings of the National Academy of Sciences* (2024). DOI: 10.1073/pnas.2406814121

Oliver Tejero et al, Active state structures of a bistable visual opsin bound to G proteins, *Nature Communications* (2024). DOI: 10.1038/s41467-024-53208-2

Provided by Paul Scherrer Institute



Discover the Power of Cold LaserTherapy for Chronic Pain Relief

- **Targeted Cold Laser Therapy at the Push of a Button:** The RedRevive™ Wand uses soothing wavelengths of cold laser light to target pain. It's designed to reduce inflammation, promote circulation, and heal damaged tissues, offering lasting relief from muscle aches, joint pain, and more.

- **Compact and Easy to Use:** RedRevive™ is designed for easy use. Just glide the wand over painful areas slowly and smoothly. Its ergonomic shape makes it easy to hold and use anywhere.
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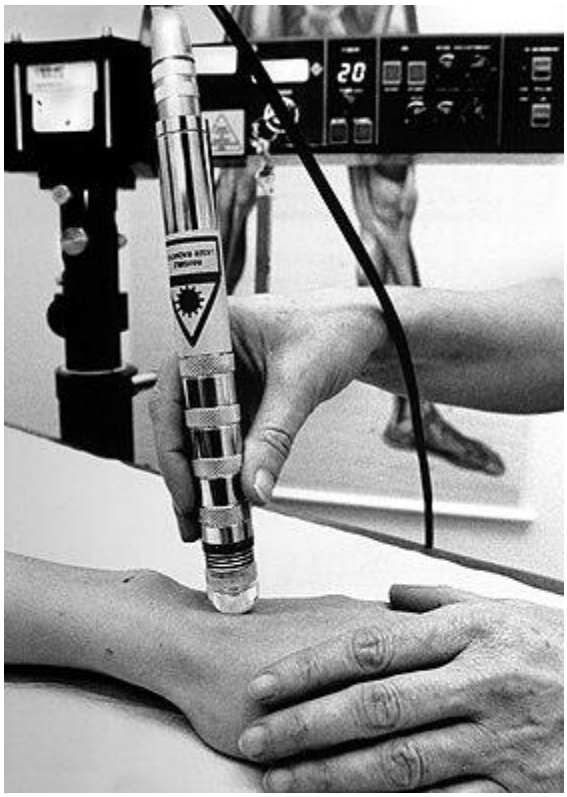
Dr. Jeff Norman, Chronic Pain Specialist

RedRevive™ is changing lives by providing a safe, reliable solution to chronic pain. Join the thousands of users who have experienced real relief!

Low-level laser therapy

Not to be confused with Light therapy.

Low-level laser therapy



LLLT being applied for rheumatism in Sweden

MeSH D028022

Low-level laser therapy (**LLLT**), **cold laser therapy**, **photobiomodulation** (**PBM**)[1][2][3][4] or **red light therapy**[5] is a form of medicine that applies low-level (low-power) lasers or light-emitting diodes (LEDs) to the surface of the body. Whereas high-power lasers are used in laser medicine to cut or destroy tissue, it is claimed that application of low-power lasers relieves pain or stimulates and enhances cell function. The effects appear to be limited to a specified set of wavelengths and new research has demonstrated effectiveness at myopia control.[6] Several such devices are cleared by the United States Food and Drug Administration (FDA), and research shows potential for treating a range of medical problems including rheumatoid arthritis[7] and oral mucositis.[8]

Mechanism

This section **needs expansion**. You can help by adding to it. *(June 2023)*

Research is ongoing about the mechanism of LLLT. The effects of LLLT appear to be limited to a specified set of wavelengths of laser,[9] and administering LLLT below the dose range does not appear to be effective.[10] Photochemical reactions are well known in biological research, and LLLT make use of the first law in photochemistry (Grotthuss-Draper law): light must be absorbed by a chemical substance in order for a photochemical reaction to take place. In LLLT that chemical substance is represented by the respiratory enzyme cytochrome c oxidase which is involved in the electron transport chain in mitochondria,[11][12] which is the generally accepted theory.

Medical uses

Various LLLT devices have been promoted for use in treatment of several musculoskeletal conditions including carpal tunnel syndrome (CTS), fibromyalgia, osteoarthritis, and rheumatoid arthritis. They have also been promoted for temporomandibular joint disorders, wound healing, smoking cessation, and tuberculosis. LLLT appears to be effective for preventing oral mucositis in recipients of a stem cell transplant with chemotherapy.[8][13] In other areas, evidence for LLLT remains conflicted. Some studies suggest that LLLT may be modestly effective in relieving short-term pain for rheumatoid arthritis,[7] osteoarthritis,[14] chronic low back pain,[15] acute and chronic neck pain,[16] tendinopathy,[9][17] and chronic joint disorders.[10] The evidence for LLLT being useful in dentistry,[18][19] and in the treatment of wound healing[20] is unclear.

Concerns have been raised in the literature about brain stimulation techniques that rely upon low-level (low-power) lasers and light-emitting diodes (LEDs). The transcranial photobiomodulation or transcranial low level light therapy is limited in neuromodulation due to several reasons:

- An excessive dose of radiation can be harmful.[21] Therefore, at adequate doses of light there may be stimulation of growth, but at high doses excessive singlet oxygen may be produced and its chemical action may be harmful to cells.[22][21]

- Regarding LED light therapy, this neurostimulation method based on the light-emitting diodes stimulation cannot pass through the skin, only laser can penetrate deeper tissues and stimulate brain areas accordingly. The penetration depth of white light and LED light into the skin increases with increasing wavelength from the UV to the visible light range, and then decreases again in the IR range depending on the selected optical properties. This depth further increases if the thickness of the stratum corneum decreases.[23] Broadband polychromatic light (white light) and LED radiation

can only penetrate 0.0017 mm to 5 mm of tissue.[24] For example, research shows that at wavelengths of 450 nm and 650 nm only 1% of the light reaches approximately 1.6 mm and very little reaches 5 mm.[25][26] Only laser radiation can propagate into deeper tissues.

- Since the action spectrum for tissue regeneration and repair consist of more than one wavelength,[27][21] laser and LED light sources may offer some disadvantages,[28] destroying healthy cells.[21] We still lack knowledge of mental processes at the cellular level. The link between neuronal activity and mental processes is still an intriguing research question and a problem in treatment targeting. Therefore, no one can be sure whether the laser beam only reaches the neuronal structures in the brain that need treatment. An undetermined dose of radiation and the target of radiation can destroy healthy cells during the treatment procedure.[21]

- **Ten Benefits of Cold Laser Therapy**
- Cold laser therapy, also known as low-level laser therapy (LLLT) or photobiomodulation, has gained recognition for its various therapeutic benefits across a range of medical and healthcare applications. Here are some of the key benefits associated with cold laser therapy:
- 1. Pain Relief: Cold laser therapy is widely used for its analgesic effects. The low-level laser light stimulates the release of endorphins, the body's natural painkillers, helping to alleviate both acute and chronic pain conditions. This makes it valuable for managing conditions such as arthritis, fibromyalgia, and musculoskeletal injuries.
- 2. Reduced Inflammation: The anti-inflammatory properties of cold laser therapy contribute to reducing swelling and inflammation. By modulating the inflammatory response at a cellular level, the therapy promotes faster healing and provides relief for conditions like tendonitis and bursitis.
- 3. Accelerated Healing: Cold laser therapy enhances cellular function and metabolism, leading to faster tissue repair and regeneration. This makes it beneficial for healing wounds, cuts, and injuries, as well as post-surgical recovery. Athletes often turn to cold laser therapy to expedite the healing of sports injuries.
- 4. Improved Blood Circulation: The therapy stimulates the production of nitric oxide, a molecule that enhances vasodilation and improves blood flow. Improved circulation is vital for delivering oxygen and nutrients to tissues, supporting overall tissue health and aiding in the recovery process.
- 5. Non-Invasive and Painless: One of the significant advantages of cold laser therapy is its non-invasive nature. Unlike surgical interventions or certain drug treatments, cold laser therapy does not involve incisions or the use of medications, making it a safe and painless option.

- 6. Minimal Side Effects: Cold laser therapy has minimal side effects. People typically experience no discomfort during or after the treatment. This is a stark contrast to some pharmaceutical interventions that may have adverse effects.
- 7. Versatility in Applications: Cold laser therapy is versatile and can be applied to various medical conditions. It is used in physical therapy, chiropractic care, dermatology, dentistry, and veterinary medicine, showcasing its adaptability across different healthcare disciplines.
- 8. Reduction in Scarring: By promoting optimal healing conditions, cold laser therapy may help minimize scar formation. This is particularly beneficial for people recovering from surgeries or injuries where scarring could be a concern.
- 9. Enhanced Nerve Function: Cold laser therapy has shown promise in improving nerve function and can be utilized in the management of conditions such as peripheral neuropathy. It aids in reducing pain associated with nerve damage and promotes neural regeneration.
- 10. Well-Tolerated : People generally find cold laser therapy sessions to be comfortable and well-tolerated. The absence of pain experienced during the treatment contributes to a positive experience.

Age Reversal Breakthrough: Harvard/MIT Discovery Could Enable Whole-Body Rejuvenation

Age Reversal Breakthrough: Harvard/MIT Discovery Could Enable Whole-Body Rejuvenation

Scientists from Harvard Medical School, the University of Maine, and MIT have published a groundbreaking study revealing a chemical method to reprogram cells to a more youthful state. This technique offers a potential alternative to gene therapy for reversing aging. The implications of this research are vast, with potential applications in regenerative medicine, treatment of age-related diseases, and whole-body rejuvenation. In a pioneering study, researchers from Harvard Medical School, University of Maine, and MIT have introduced a chemical method for reversing cellular aging. This revolutionary approach offers a potential alternative to gene therapy for age reversal. The findings could

transform treatments for age-related diseases, enhance regenerative medicine, and potentially lead to whole-body rejuvenation.

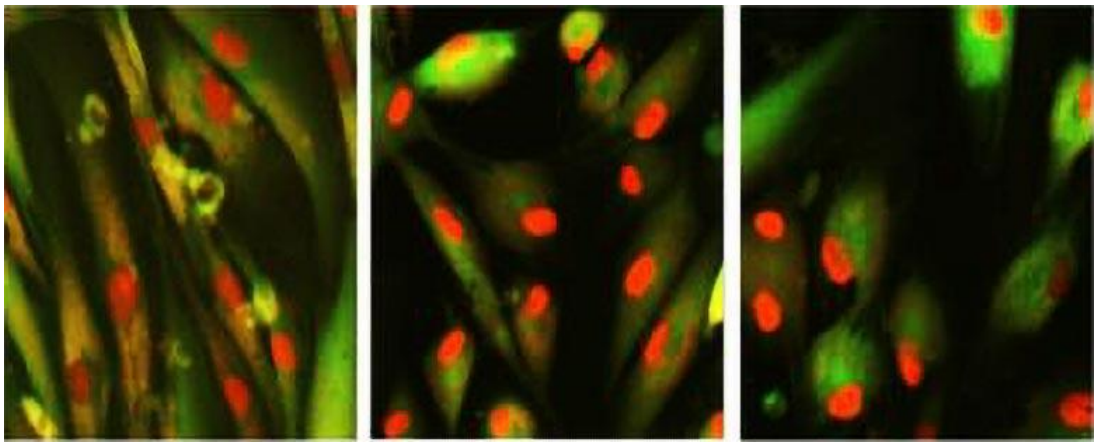
Groundbreaking Discovery in Aging Reversal

In a monumental study, a team of researchers has revealed a novel approach to combating aging and age-related diseases. This work, undertaken by scientists at Harvard Medical School, introduces the first chemical method to rejuvenate cells, bringing them to a more youthful state. Prior to this, only powerful gene therapy could achieve this feat. Mice in the Sinclair lab have been engineered to age rapidly to test the effectiveness of therapies to reverse the aging process. The mouse on the right has been aged to 150% that of its sibling on the left by disrupting its epigenome. Photo credit: D. Sinclair, Harvard Medical School. Credit: 2023 Yang et al.

On July 12, 2023, researchers from Harvard Medical School, the University of Maine, and the Massachusetts Institute of Technology (MIT) published a fresh research paper in *Aging*. The paper, titled, “Chemically induced reprogramming to reverse cellular aging,” extends upon a previously groundbreaking discovery. The researchers are Jae-Hyun Yang, Christopher A. Petty, Thomas Dixon-McDougall, Maria Vina Lopez, Alexander Tyshkovskiy, Sun Maybury-Lewis, Xiao Tian, Nabilah Ibrahim, Zhili Chen, Patrick T. Griffin, Matthew Arnold, Jien Li, Oswaldo A. Martinez, Alexander Behn, Ryan Rogers-Hammond, Suzanne Angeli, Vadim N. Gladyshev, and David A. Sinclair.

Exploring the Methodology

This discovery builds on the finding that the expression of specific genes, known as Yamanaka factors, can transform adult cells into induced pluripotent stem cells (iPSCs). This breakthrough, which earned a Nobel Prize, prompted scientists to question if cellular aging could be reversed without pushing cells to become too young and potentially cancerous.



Rejuvenation and age reversal of senescent human skin cells by chemical means. Cells in the right two panels have restored compartmentalization of the red fluorescent protein in the nucleus, a marker of youth that was used to find the cocktails, before the scientists

confirmed they were younger, based on how genes were expressed. Image credit: J. -H. Yang, Harvard Medical School. Credit: 2023 Yang et al.

In this recent study, the scientists probed for molecules that could, in tandem, revert cellular aging and refresh human cells. They designed advanced cell-based assays to differentiate between young and old, as well as senescent cells. The team employed transcription-based aging clocks and a real-time nucleocytoplasmic protein compartmentalization (NCC) assay. In a significant development, they identified six chemical combinations that could return NCC and genome-wide transcript profiles to youthful states, reversing transcriptomic age in less than a week.

Relevance and Potential Applications

The Harvard team has previously shown the possibility of reversing cellular aging without causing unregulated cell growth. This was done by inserting specific Yamanaka genes into cells using a viral vector. Studies on various tissues and organs like the optic nerve, brain, kidney, and muscle have yielded encouraging results, including improved vision and extended lifespan in mice. Additionally, recent reports have documented improved vision in monkeys.

These findings have profound implications, paving the way for regenerative medicine and potentially full-body rejuvenation. By establishing a chemical alternative to gene therapy for age reversal, this research could potentially transform the treatment of aging, injuries, and age-related diseases. The approach also suggests the possibility of lower development costs and shorter timelines. Following successful results in reversing blindness in monkeys in April 2023, plans for human clinical trials using the lab's age reversal gene therapy are currently underway.

Views from the Research Team

"Until recently, the best we could do was *slow* aging. New discoveries suggest we can now reverse it," said David A. Sinclair, A.O., Ph.D., Professor in the Department of Genetics and co-Director of the Paul F. Glenn Center for Biology of Aging Research at Harvard Medical School and lead scientist on the project. "This process has previously required gene therapy, limiting its widespread use."

The team at Harvard envisions a future where age-related diseases can be effectively treated, injuries can be repaired more efficiently, and the dream of whole-body rejuvenation becomes a reality. "This new discovery offers the potential to reverse aging with a single pill, with applications ranging from improving eyesight to effectively treating numerous age-related diseases," Sinclair said.

Reference: "Chemically induced reprogramming to reverse cellular aging" by Jae-Hyun Yang, Christopher A. Petty, Thomas Dixon-McDougall, Maria Vina Lopez, Alexander Tyshkovskiy, Sun Maybury-Lewis, Xiao Tian, Nabilah Ibrahim, Zhili Chen, Patrick T. Griffin, Matthew Arnold, Jien Li, Oswaldo A. Martinez, Alexander Behn, Ryan Rogers-

Hammond, Suzanne Angeli, Vadim N. Gladyshev and David A. Sinclair, 12 July 2023, *Aging-US*.
DOI: 10.18632/aging.204896

Published: 21 October 2019

Integrated photonic quantum technologies

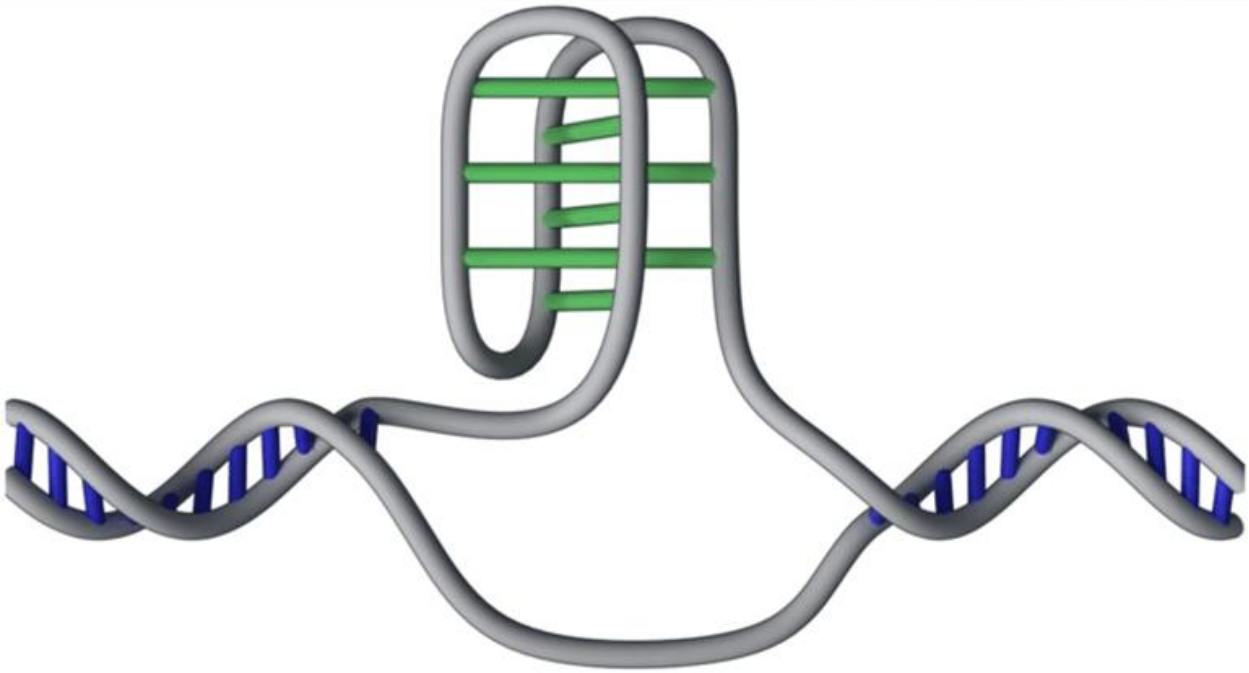
- Jianwei Wang,
- Fabio Sciarrino,
- Anthony Laing &
- Mark G. Thompson

Nature Photonics **volume 14**, pages273–284 (2020)Cite this article

Abstract

Quantum technologies comprise an emerging class of devices capable of controlling superposition and entanglement of quantum states of light or matter, to realize fundamental performance advantages over ordinary classical machines. The technology of integrated quantum photonics has enabled the generation, processing and detection of quantum states of light at a steadily increasing scale and level of complexity, progressing from few-component circuitry occupying centimetre-scale footprints and operating on two photons, to programmable devices approaching 1,000 components occupying millimetre-scale footprints with integrated generation of multiphoton states. This Review summarizes the advances in integrated photonic quantum technologies and its demonstrated applications, including quantum communications, simulations of quantum chemical and physical systems, sampling algorithms, and linear-optic quantum information processing.

There Are 50,000 Knots In Your DNA And They've Just Been Mapped



Sometimes DNA does not follow the usual rules, with cytosines on a strand connecting to each other, rather than guanines on the other strand. - Image credit: Garvan Institute© IFL Science

The locations of "knot-like structures" in the human genome have been mapped for the first time. Known as i-motifs, these odd shapes were reported to exist in 1993, but initially treated with suspicion. The discovery of how frequent they are, and their locations, suggests they play important roles in our health, but can also lead to disease.

The double helix structure of DNA is so famous it serves as a sort of visual shorthand. However, in the 1990s geneticists started to suspect it is sometimes interrupted by secondary structures they called i-motifs, whose existence outside the lab was only confirmed in 2018.

Specifically, instead of the four bases from which DNA is constructed linking to each other like normal (adenine to thymine on the opposite strand, cytosine to guanine) cytosines on the same strand hook up. The result is a brief section that sticks out of the double helix as a four-stranded structure.

Professor Daniel Christ of the Garvan Institute of Medical Research told IFLScience, "I-motifs were first discovered in the laboratory under test tube conditions (in vitro conditions). These conditions were somewhat different (more acidic) from those observed inside cells. This had initially raised questions as to whether i-motifs existed in cells. However, once we had generated a specific antibody for the i-motif structure, we and others were able to demonstrate they do exist in human cells." Christ added that around 25,000 i-motifs have been found in rice by another team, so it is definitely something common across very different organisms.

Certain patterns of the four bases have been found to be more prone to forming i-motifs, but scientists have remained unsure of why they exist or how common they are. Now Christ's team have mapped the i-motifs on the human genome and found they're surprisingly common.

"In this study, we mapped more than 50,000 i-motif sites in the human genome that occur in all three of the cell types we examined," Christ said in a statement. "That's a remarkably high number for a DNA structure whose existence in cells was once considered controversial. Our findings confirm that i-motifs are not just laboratory curiosities but widespread – and likely to play key roles in genomic function."

What those functions are will take a long time to resolve, but the locations of the i-motifs offer some clues. "We discovered that i-motifs are associated with genes that are highly active during specific times in the cell cycle. This suggests they play a dynamic role in regulating gene activity," said first author Cristian David Peña Martinez, but Christ indicated to IFLScience the specifics remain unclear.

"We also found that i-motifs form in the promoter region of oncogenes, for instance the *MYC* oncogene, which encodes one of cancer's most notorious 'undruggable' targets," Peña Martinez added. Potentially then, a way to attack cancers that are currently particularly resistant to treatment could be through making i-motifs unravel. If, as seems likely, they serve a function elsewhere in the DNA, this might need to be very carefully targeted, however. Nevertheless, co-author Dr Sarah Kummerfeld said, "It might be possible to design drugs that target i-motifs to influence gene expression, which could expand current treatment options."

If i-motifs formed in response to specific conditions, such as stress in the womb, this could be another non-genetic way we are affected by our environment. However, Christ told IFLScience, "There is currently no data indicating non-hereditary components, and we observed similar number of i-motifs among three human cell lines of different origin, indicating relatively broad conservation. "

The study is published in The EMBO Journal.

Published: 12 May 2022

Extremely low-frequency pulses of faint magnetic field induce mitophagy to rejuvenate mitochondria

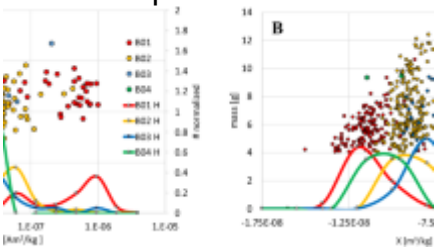
- Takuro Toda,
- Mikako Ito,
- Jun-ichi Takeda,
- Akio Masuda,
- Hiroyuki Mino,
- Nobutaka Hattori,
- Kaneo Mohri &
- Kinji Ohno

Communications Biology **volume 5**, Article number: 453 (2022) Cite this article

Abstract

Humans are frequently exposed to time-varying and static weak magnetic fields (WMF). However, the effects of faint magnetic fields, weaker than the geomagnetic field, have been scarcely reported. Here we show that extremely low-frequency (ELF)-WMF, comprised of serial pulses of 10 μT intensity at 1–8 Hz, which is three or more times weaker than the geomagnetic field, reduces mitochondrial mass to 70% and the mitochondrial electron transport chain (ETC) complex II activity to 88%. Chemical inhibition of electron flux through the mitochondrial ETC complex II nullifies the effect of ELF-WMF. Suppression of ETC complex II subsequently induces mitophagy by translocating parkin and PINK1 to the mitochondria and by recruiting LC3-II. Thereafter, mitophagy induces PGC-1α-mediated mitochondrial biogenesis to rejuvenate mitochondria. The lack of PINK1 negates the effect of ELF-WMF. Thus, ELF-WMF may be applicable for the treatment of human diseases that exhibit compromised mitochondrial homeostasis, such as Parkinson's disease.

Article Open access19 June 2021



Magnetic domains oscillation in the brain with neurodegenerative disease

Article Open access12 January 2021

Introduction

In the present-day industrialized societies, humans are exposed daily to time-varying and static weak magnetic fields (WMF). The effects of WMF on animals, including humans, have been documented in a few reports. WMF increases intracellular calcium concentrations and induces the development of satellite cells[1]. Similarly, static magnetic fields increase cytosolic calcium and reactive oxygen species (ROS) in mouse embryonic stem (ES) cell-derived embryoid bodies and Flk-1+ cardiac progenitor cells[2], although the magnetic intensities were as much as 0.3–5.0 mT. In addition, static WMF as weak as 0.01 μT reduces the ROS level in nonactivated neutrophils[3]. Moreover, the exposure to static WMF of 200–600 μT in HT1080 cells increased the mitochondrial calcium concentration and the mitochondrial membrane potential[4]. For a disease model, the viability of breast cancer cells is specifically decreased by WMF[5]. Extremely low-frequency WMF (ELF-WMF), which is defined as ELF with a frequency of 300 Hz or less, may or may not reduce the levels of ROS in cells[6]. ROS are mostly produced during electron transfer through the mitochondrial electron transport chain (ETC). The biological effects of ELF-WMF, weaker than the geomagnetic field, have been reported in cultured cells[7], planaria[8], rats[9], lizards[10,11], and humans[12], but the underlying mechanisms remain elusive. In addition, the optimal conditions for the manifestation of the cellular effects of ELF-WMF remain undetermined. Furthermore, the molecular mechanisms underlying the effects of ELF-WMF have not been elucidated.

Mitophagy and mitochondrial biogenesis cooperate in the maintenance of mitochondrial homeostasis. Mitophagy is a quality-assurance system that selectively eliminates damaged mitochondria using the macroautophagy machinery. Mitophagy-associated proteins include PTEN-induced kinase 1 (PINK1), parkin, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like (NIX/BNIP3L), and FUN14 domain containing 1 (FUNDC1). Mitophagy is induced by mitochondrial damage, excessive levels of mitochondrial ROS, endoplasmic reticulum (ER) stress[13], circadian rhythm[14], and hypoxia-inducible factor[15]. In addition, mitophagy induces mitochondrial biogenesis to compensate for the removal of mitochondria[16,17]. The parkin/PINK1 pathway is a key regulator of mitophagy. Parkin and PINK1 translocate from the cytosol to the mitochondria, and ubiquitinate the mitochondria, which are subsequently recognized by the phagosome-lining LC3 to eliminate the mitochondria. Accumulating knowledge points to the notion that the compromised parkin/PINK1 pathway is associated with the development and progression of neurodegenerative diseases, including Parkinson's disease[18] and Alzheimer's disease[19].

In this study, we investigated the effects of faint magnetic fields, weaker than the geomagnetic field, on cells. We report that ELF-WMF efficiently suppresses the mitochondrial mass to 70% by inhibiting the mitochondrial ETC complex II, which subsequently induces mitophagy and rejuvenates mitochondria. We expect that ELF-WMF may be applicable to a plethora of human diseases that exhibit compromised mitophagy like neurodegenerative diseases.

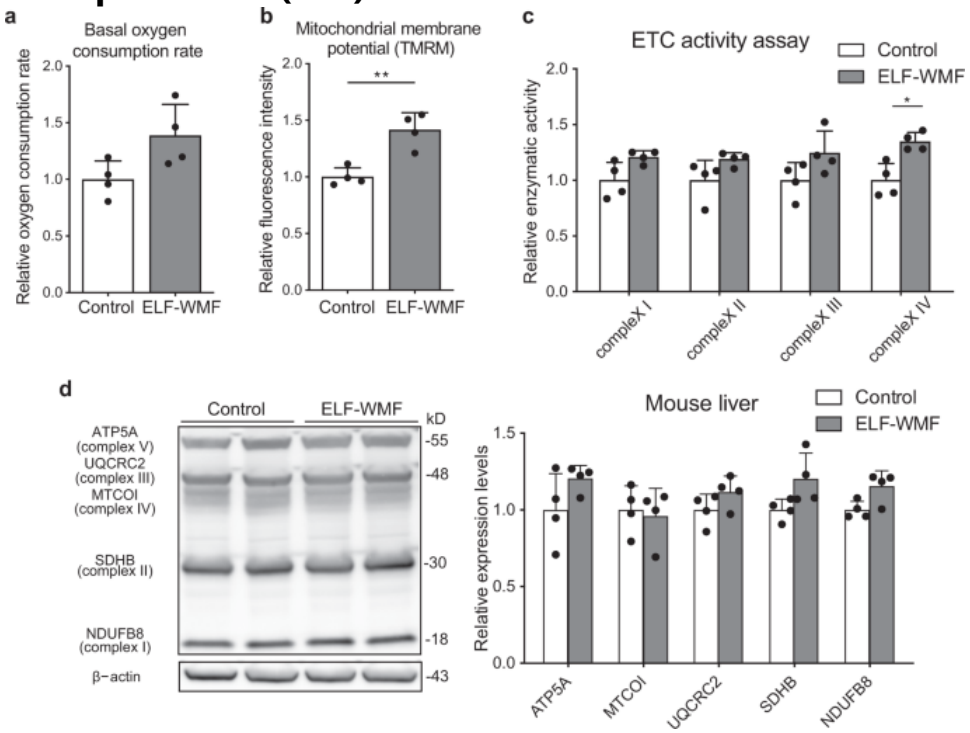
Results

Exposure to Opti-ELF-WMF for 4 weeks increases the mitochondrial activity in the mouse liver

Based on our previous observation that 1–8 Hz stimulation of 4 ms pulses of 10 μT magnetic field (Opti-ELF-WMF) decreases the thermal hysteresis of electric resistance of modified Ringer's solution[20,21], we examined the effect of 4-week exposure to Opti-ELF-WMF on the liver mitochondria in wild-type C57BL6/N mice. Using an open-field locomotor test, we first confirmed that exposure to Opti-ELF-WMF had no effect on the locomotor activity in mice

(Supplementary Fig. 1e, f). We examined the ETC activity of mitochondria isolated from the mouse liver by measuring the oxygen consumption rate (OCR) and mitochondrial membrane potential using a flux analyzer and tetramethylrhodamine (TMRM), respectively. We found that Opti-ELF-WMF increased both the OCR and mitochondrial membrane potential by approximately 40% (Fig. 1a, b).

Fig. 1: Exposure to Opti-ELF-WMF for 4 weeks increased mitochondrial electron transport chain (ETC) activities in the mouse liver.



a Basal oxygen consumption rate was measured by a flux analyzer using mitochondria isolated from the mouse liver. No statistically significant difference was observed by Student's *t*-test (mean ± SD, *n* = 4 mice each). **b** Membrane potential of mitochondria isolated from the mouse liver was measured by flow cytometry with tetramethylrhodamine (TMRM; mean ± SD, *n* = 4 mice each; $^{**}p < 0.01$ by Student's *t*-test). **c** Relative enzymatic activities of mitochondrial electron transport chain (ETC) complexes I, II, III, and IV of the mouse liver (mean ± SD, *n* = 4 mice each; $^{*}q$ [false discovery rate] < 0.05 by multiple Student's *t*-tests). **d** Western blotting of the mitochondrial oxidative phosphorylation proteins in the mouse liver (mean ± SD *n* = 4 mice each; no statistical difference was observed by false

discovery rate with multiple Student's *t*-tests). Representative duplicates of Western blot analysis are shown. See also Fig. S1.

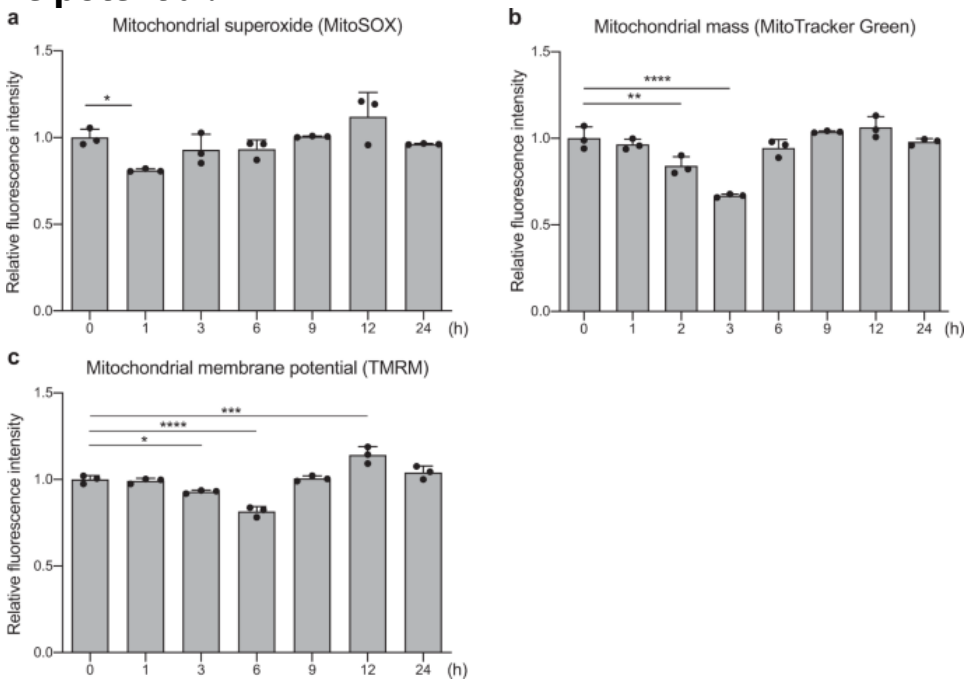
Next, we evaluated the enzymatic activity of each ETC complex and the amount of OXPHOS proteins in the mouse liver homogenates. Opti-ELF-WMF increased the activities of mitochondrial ETC complexes I to IV, although statistical significance was observed only in complex IV (Fig. 1c). The levels of four nucleus-encoded proteins (NDUFB8 [complex I], SDHB [complex II], UQCRC2 [complex III], and ATP5F1A [complex V]) also tended to be increased (Fig. 1d). In contrast, the levels of mitochondria-encoded MTCO1 [complex IV] remained unchanged.

These data demonstrate that Opti-ELF-WMF had no effect on the locomotor activity in wild-type mice, but tended to increase the mitochondrial ETC complex activities and the levels of nucleus-encoded ETC proteins in the mouse liver.

Opti-ELF-WMF temporarily decreases the mitochondrial ROS levels, mitochondrial mass, and mitochondrial membrane potential in cultured cells

To further dissect the effect of Opti-ELF-WMF on the mitochondria, mouse hepatocyte-derived AML12 cells were cultured under Opti-ELF-WMF for 1 to 24 h, and were stained with MitoSOX, MitoTracker Green, and TMRM to quantify the levels of mitochondrial superoxide, mitochondrial mass, and mitochondrial membrane potential, respectively. Opti-ELF-WMF most strongly decreased the level of mitochondrial superoxide at 1 h, mitochondrial mass at 3 h, and mitochondrial membrane potential at 6 h, and most strongly increased them at 12 h (Fig. 2a–c). At 24 h, the values reverted to normal levels. Thus, Opti-ELF-WMF suppressed the mitochondrial ETC activity at 1 h, which was likely to be followed by elimination and/or inactivation of a subset of mitochondria at 3 to 6 h. The mass and function of mitochondria were then increased at 12 h and returned to normal levels at 24 h. These time points were used for subsequent analyses.

Fig. 2: Opti-ELF-WMF temporarily decreased the levels of mitochondrial reactive oxygen species (ROS), mitochondrial mass, and mitochondrial membrane potential.



a Mitochondrial ROS of mouse hepatocyte-derived AML12 cells exposed to Opti-ELF-WMF for 1 to 24 h was evaluated by MitoSOX. **b** Mitochondrial mass of AML12 cells exposed to Opti-ELF-WMF for 1 to 24 h was evaluated by MitoTracker Green. **c** Mitochondrial membrane potential of AML12 cells exposed to Opti-ELF-WMF for 1 to 24 h was evaluated by tetramethylrhodamine (TMRM). **a**–**c** Show mean ± SD, $n = 3$ culture dishes each; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ by one-way ANOVA followed by Dunnett's post hoc test compared with the value at time 0.

Optimal conditions of ELF-WMF for the reduction of the mitochondrial mass in cultured cells

Next, we analyzed the optimal conditions of ELF-WMF by measuring the mitochondrial mass at 3 h by changing the intensity, pulse width, and frequency of ELF-WMF. ELF-WMF, less than 10 μT, showed MF intensity-dependent reduction in the mitochondrial mass, but the effects were not enhanced when the MF intensity ranged from 10 to 200 μT (Supplementary Fig. 2a). However, compared to 10 μT, 300 μT ELF-WMF had a marginally

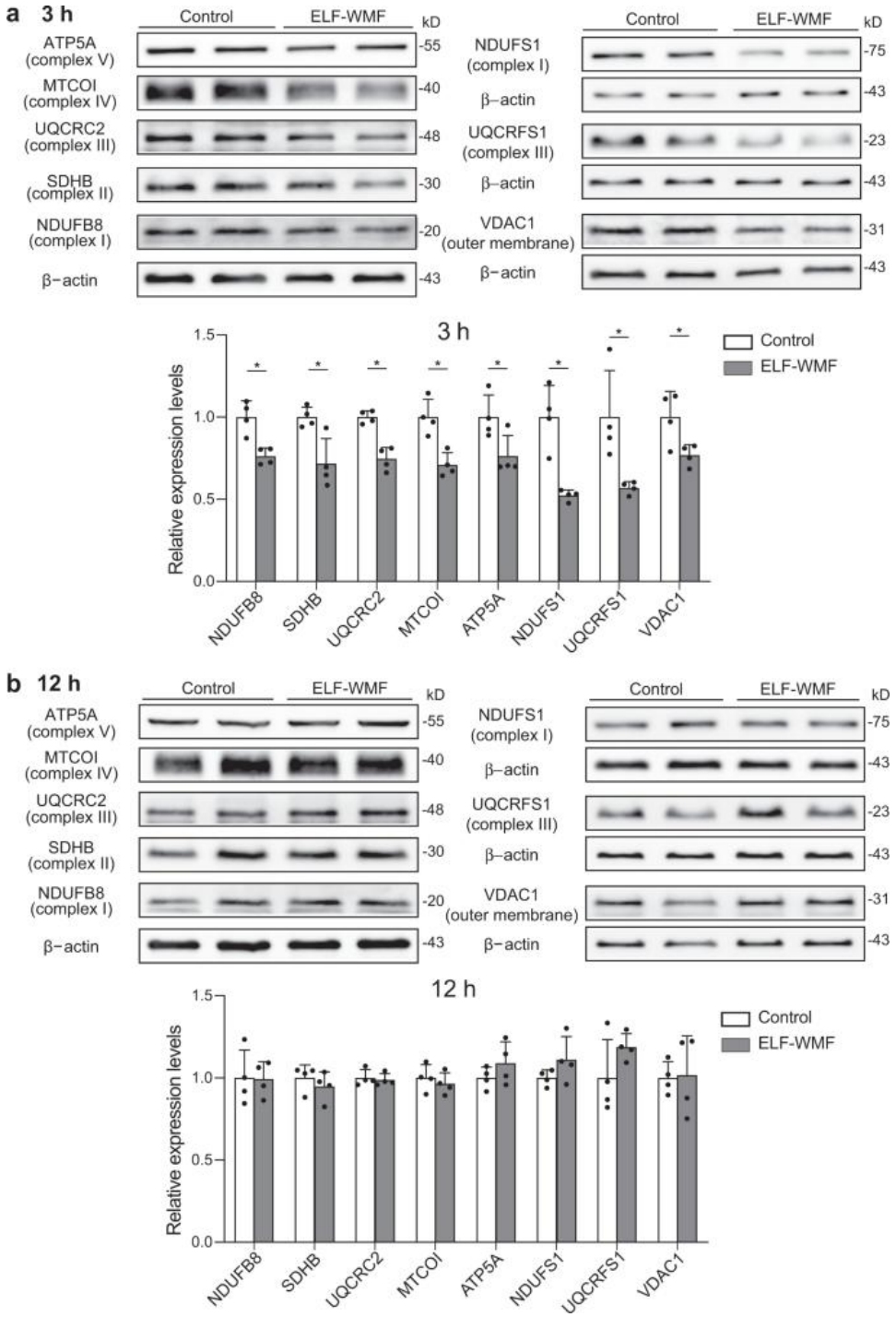
reduced effect. ELF-WMF with pulse widths of 2, 4, and 8 ms reduced the mitochondrial mass, with a peak at 4 ms (Supplementary Fig. 2b). In contrast, ELF-WMF with pulse widths of 1 and 16 ms had no effect. The mitochondrial mass was reduced the most upon treatment with increasing frequencies of ELF-WMF (1, 2, 3, 4, 5, 6, 7, and 8 Hz for 1 s each) (Supplementary Fig. 2c). Static frequencies at 6 and 8 Hz had no effect. Similarly, changing the frequency profiles to 1–4 Hz for 1 s each or to 1–16 Hz for 1 s each had no effect.

As observed for AML12 cells (Fig. 2b, c), Opti-ELF-WMF first reduced the mitochondrial mass, and thereafter increased the mitochondrial membrane potential in Neuro2A, C2C12, human iPS, HEK293, and HeLa cells (Supplementary Table 2). Thus, the effects of Opti-ELF-WMF on mitochondria are unlikely to be cell line-specific.

Decrease in the mitochondrial mass by Opti-ELF-WMF is accounted for by temporary decreases in the levels of mitochondrial ETC proteins and of outer membrane proteins

To identify the mitochondrial proteins that were decreased by the Opti-ELF-WMF exposure, we quantified the amounts of mitochondrial ETC proteins and VDAC1, which is an outer membrane protein, by Western blot analysis. Opti-ELF-WMF had no effect on the levels of the eight examined mitochondrial proteins at 1 h (Supplementary Fig. 3), but decreased them at 3 h (Fig. 3). At 12 h, the amounts of five proteins (NDUFB8, SDHB, UQCRC2, MTCO1, and VDAC1) were restored to their basal levels, and those of three proteins (ATP5A, NDUFS1, and UQCRFS1) were increased compared to their basal levels. Taken together, Opti-ELF-WMF decreased the levels of all the examined mitochondrial proteins at 3 h, which was consistent with the decreased mitochondrial mass at 3 h after exposure (Fig. 2b). The levels of mitochondrial proteins were restored to normal or higher than normal levels at 12 h.

Fig. 3: Temporary downregulation of the levels of mitochondrial proteins at 3 h and recovery or slight increase at 12 h in cells exposed to Opti-ELF-WMF.



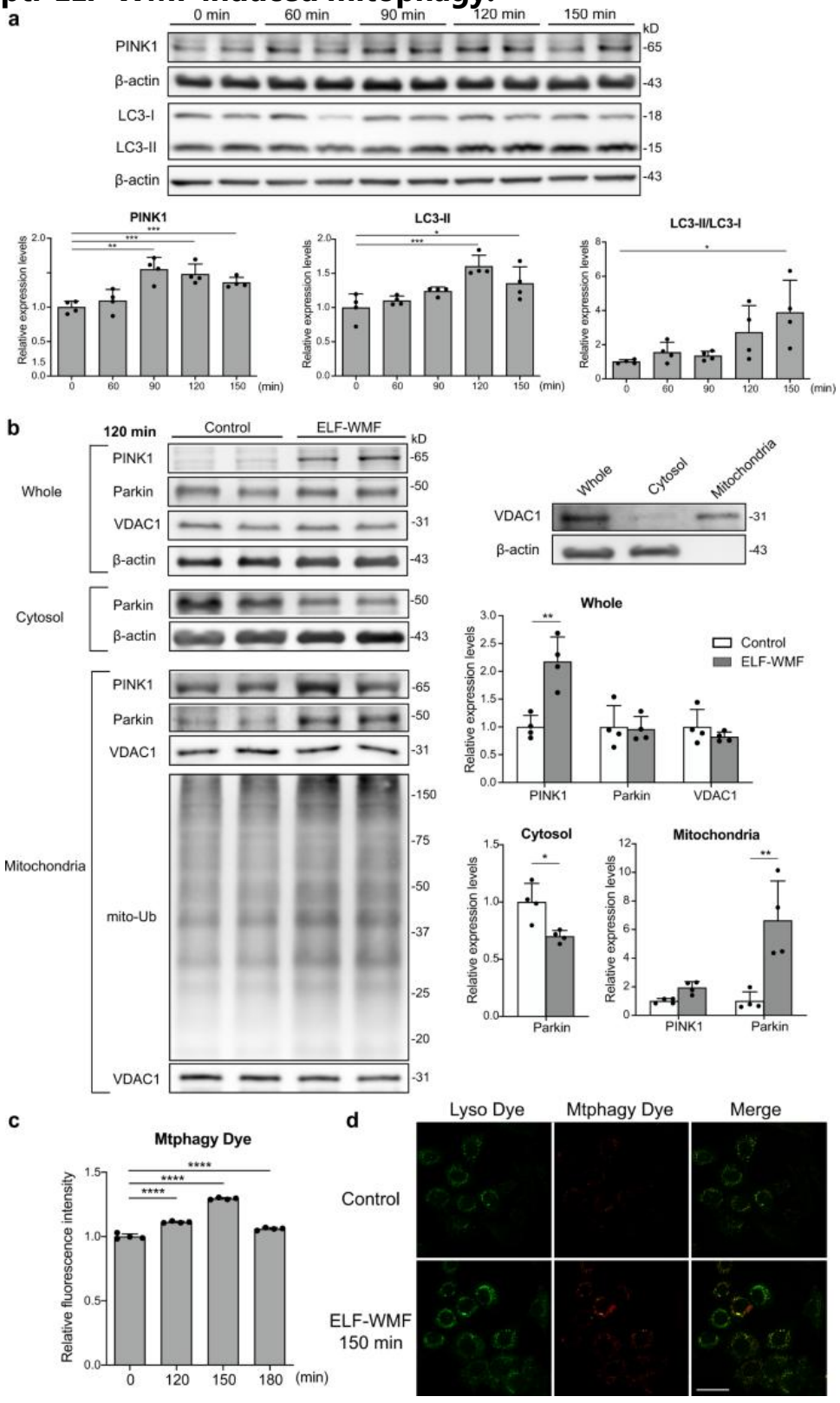
Representative duplicates of Western blot analysis of mitochondrial oxidative phosphorylation proteins, as well as of a mitochondrial outer membrane protein, VDAC1, in AML12 cells are shown at 3 h (**a**) and 12 h (**b**) exposure to

Opti-ELF-WMF (mean ± SD, $n = 4$ culture dishes each; *$q < 0.05$, and **$q < 0.01$ by multiple Student's t-tests). See also Fig. S3.

Opti-ELF-WMF induces mitophagy

Mitophagy is an autophagic mechanism in the mitochondrial quality assurance system that eliminates damaged mitochondria. We investigated whether mitophagy is activated by Opti-ELF-WMF. We first examined the expression levels of mitophagy-related proteins, PINK1 and LC3-II, in whole cell lysates of AML12 cells. PINK1 triggers mitophagy, whereas LC3-II is an effector that eliminates the mitochondria. The amount of PINK1 gradually increased until 90 min and gradually decreased thereafter upon exposure to Opti-ELF-WMF (Fig. 4a). Similarly, the amount of LC3-II gradually increased until 120 min and gradually decreased thereafter (Fig. 4a). These results indicated that the decrease in mitochondrial mass by Opti-ELF-WMF was likely due to the activation of mitophagy.

Fig. 4: Opti-ELF-WMF induced mitophagy.



a AML12 cells were exposed to Opti-ELF-MF for up to 150 min. The levels of PINK1 and LC3-II were evaluated by Western blot analysis (mean ± SD, $n = 4$ culture dishes each; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ by one-way ANOVA followed by Dunnett's post hoc test compared with the value at time 0). **b** Western blot analysis of β-actin and VDAC1 in whole, cytosolic, and mitochondrial fractions of AML12 cells to indicate the purity of each fraction. Representative duplicated Western blot analysis for parkin and PINK1 in whole cell lysates, a cytosolic fraction, and a mitochondrial fraction, as well as for ubiquitination in a mitochondrial fraction of AML12 cells exposed to Opti-ELF-WMF for 120 min (mean ± SD, $n = 4$ culture dishes each; *$q < 0.05$ and **$q < 0.01$ by multiple Student's t-tests). **c** Mitophagy was evaluated using Mtphagy Dye in AML12 cells exposed to Opti-ELF-WMF for up to 180 min (mean ± SD, $n = 4$ culture dishes each; ****$p < 0.0001$ by one-way ANOVA followed by Dunnett's posthoc test compared with the value at time 0). **d** Representative confocal images showing colocalization of mitochondria, detected by Mtphagy Dye, and lysosomes, detected by Lyso Dye, in AML12 cells exposed to Opti-ELF-WMF for 150 min. Scale bar, 50 μm. See also Fig. S4.

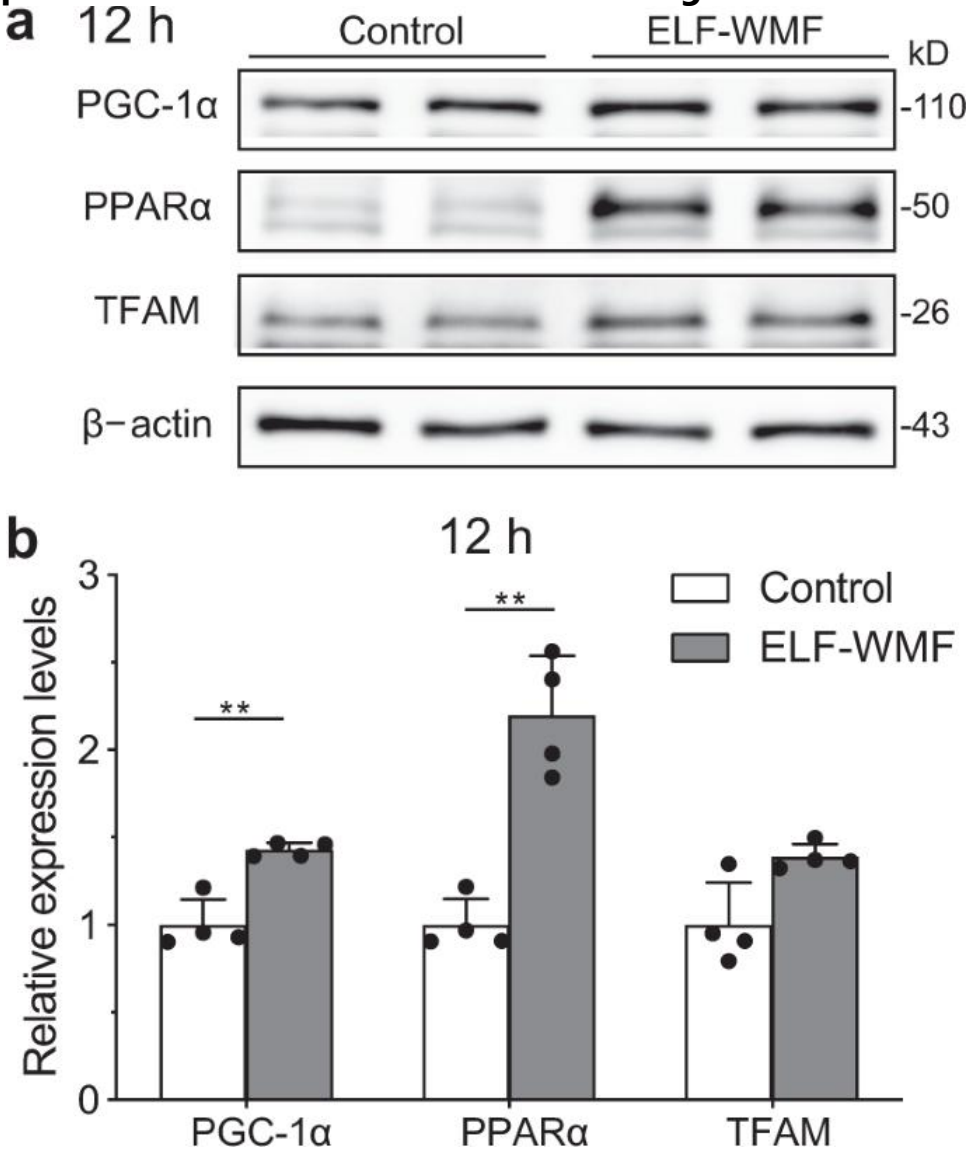
Next, we isolated the mitochondrial and cytosolic fractions of AML12 cells, and examined the purity by immunoblotting of β-actin and VDAC1, respectively (Fig. 4b). We evaluated the expression levels of PINK1 and parkin, and mitochondrial ubiquitination at 120 min in the mitochondrial and cytosolic fractions. PINK1 and parkin translocate from the cytosol to the mitochondria, and ubiquitinate mitochondrial proteins. Opti-ELF-WMF increased the level of parkin in the mitochondria, but had no effect in the whole cells (Fig. 4b). In contrast, Opti-ELF-WMF increased the levels of PINK1 in both mitochondria and whole cells (Fig. 4b). We also found that Opti-ELW-WMF induced the ubiquitination of mitochondrial proteins (Fig. 4b). These results indicated that Opti-ELF-WMF accumulated PINK1 and parkin in the mitochondria, and induced mitochondrial ubiquitination. To detect the mitochondria in the lysosomes, we used the Mtphagy Dye that fluoresces with decreasing pH around mitochondria. The fluorescence intensity of the Mtphagy Dye peaked at 150 min (Fig. 4c). We also confirmed the colocalization of mitochondria and lysosomes at 150 min by confocal microscopy (Fig. 4d).

To further confirm the effect of PINK1 on Opti-ELF-WMF-induced mitophagy, we examined the mitochondrial mass and the amounts of ATP5A and VDAC1 in PINK1-knocked out (KO) HeLa cells[22]. As expected, exposure of PINK1-KO HeLa cells to Opti-ELF-WMF failed to reduce the mitochondrial mass (Supplementary Fig. 4a), or the levels of mitochondrial proteins (Supplementary Fig. 4b).

PGC-1α expression is upregulated for mitochondrial biogenesis after mitophagy

PGC-1α is a key player in mitochondrial biogenesis. PPARα and TFAM are regulated by PGC-1α, and are effectors of mitochondrial biogenesis and metabolism. Thus, we examined whether the recovery of mitochondrial mass was mediated by PGC-1α, TFAM, and PPARα. We observed that Opti-ELF-WMF increased the expression of these proteins at 12 h (Fig. 5), indicating that PGC-1α-mediated mitochondrial biogenesis was activated after mitophagy to rejuvenate mitochondria.

Fig. 5: Opti-ELF-WMF induced mitochondrial biogenesis.



a Representative duplicates of Western blot analysis are shown. **b** Densitometric analysis of Western blots (mean ± SD, $n = 4$ culture dishes each; *$q < 0.05$ and **$q < 0.01$ by multiple Student's t-tests).

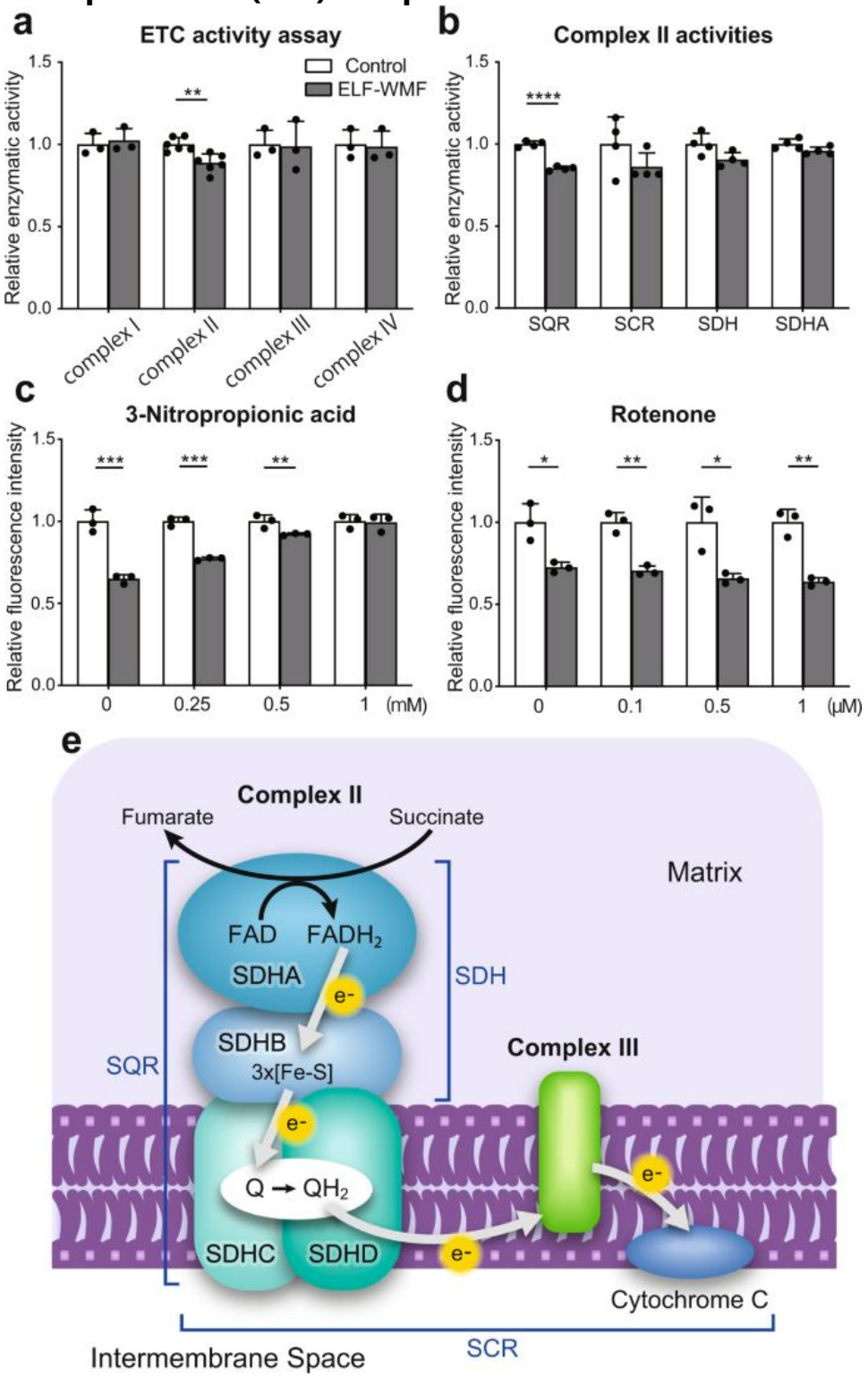
Opti-ELF-WMF suppresses the enzymatic activity of ETC complex II in vitro

We conducted RNA-sequence (RNA-seq) analysis along with gene set enrichment analysis (GSEA) using AML12 cells exposed to Opti-ELF-WMF for 1 h. We found that Opti-ELF-WMF reduced the expression of mitochondrial ETC genes (Table 1). Thus, suppression of mitochondrial ETC genes is likely to be a key factor in the triggering of mitophagy by Opti-ELF-WMF.

Table 1 Gene Ontologies (GOs) suppressed by Opti-ELF-WMF in mouse hepatocyte-derived AML12 cells at 1 h by Gene Set Enrichment Analysis (GSEA).

To capture the initial event activated by Opti-ELF-WMF, we examined the direct effects of Opti-ELF-WMF on the enzymatic activities of mitochondrial ETC complexes I, II, III, and IV in mouse liver homogenates that were exposed to Opti-ELF-WMF for 8 min in vitro. The enzymatic activity of ETC complex II was reduced to 88% by Opti-ELF-WMF, whereas the activities of the other ETC complexes (I, III, and IV) remained unchanged (Fig. 6a). Mitochondrial ETC complex II is comprised of four succinate dehydrogenase (SDH) subunits: SDHA, SDHB, SDHC, and SDHD (Fig. 6e). To further dissect the effect of Opti-ELF-WMF on ETC complex II, we quantified the enzymatic activities of succinate:quinone reductase (SQR), succinate cytochrome *c* reductase (SCR), SDH, and SDHA in the mitochondria isolated from mouse liver homogenates exposed to Opti-ELF-WMF for 8 min. The activities of SQR, SCR, SDH, and SDHA decreased to 85%, 85%, 90%, and 95%, respectively (Fig. 6b). Thus, ELF was likely to suppress all the four subunits of mitochondrial ETC complex II. We also examined the expression of SDHA, SDHB, SDHC, and SDHD under Opti-ELF-WMF and did not find any change in the expression levels of these subunits at 1 h after exposure (Supplementary Fig. 5).

Fig. 6: Opti-ELF-WMF suppressed the activities of the mitochondrial electron transport chain (ETC) complex II.



a Relative enzymatic activities of mitochondrial ETC complex I, II, III, and IV of mouse liver homogenates exposed to Opti-ELF-WMF in vitro for 8 min

(mean ± SD, n = 3 to 6 mice each; **q < 0.01 by multiple Student's t-tests). **b** Fractional and extended ETC complex II activities (succinate:quinone reductase [SQR], succinate-cytochrome c reductase [SCR], succinate dehydrogenase [SDH], succinate dehydrogenase flavoprotein subunit [SDHA]) of isolated mitochondria exposed to Opti-ELF-WMF in vitro for 8 min (mean ± SD, n = 4 mice each; ****q < 0.0001 by multiple Student's t-tests). See **e** for a schematic of the fractional enzymatic activities. **c** Mitochondrial mass (MitoTracker Green) of AML12 cells treated with variable concentrations of a mitochondrial ETC complex II inhibitor, 3-nitropropionic acid, exposed to Opti-ELF-WMF *in cellulo* (mean ± SD, n = 3 culture dishes each; **q < 0.01 and ***q < 0.001 by multiple Student's t-tests). **d** Mitochondrial mass (MitoTracker Green) of AML12 cells treated with variable concentrations of a mitochondrial ETC complex I inhibitor, rotenone, exposed to Opti-ELF-WMF *in cellulo* (mean ± SD, n = 3 culture dishes each; *q < 0.05, and **q < 0.01 by multiple Student's t-tests). **e** Schematic to indicate the fractional and extended enzymatic activities of mitochondrial ETC complex II measured in **b**. Complex II is composed of SDHA, SDHB, SDHC, and SDHD. See also Fig. S5.

To examine whether mitophagy by Opti-ELF-WMF was indeed due to the suppression of mitochondrial ETC complex II, AML12 cells were incubated with either 3-nitropropionic acid (3-NP), an ETC complex II inhibitor, or rotenone, an ETC complex I inhibitor, for 12 h. The cells were then exposed to Opti-ELF-WMF for 3 h. Inhibition of ETC complex II by 3-NP negated the reduction in the mitochondrial mass induced by Opti-ELF-WMF (Fig. 6c). In contrast, inhibition of ETC complex I had no effect on the reduction in the mitochondrial mass induced by Opti-ELF-WMF (Fig. 6d). Taken together, Opti-ELF-WMF-mediated mitophagy requires electron flow through mitochondrial ETC complex II.

Discussion

We found that Opti-ELF-WMF reduced the amount of mitochondria by ~30% (Fig. 2b) by inhibiting mitochondrial ETC complex II by ~15% (Fig. 6a). This subsequently induced mitophagy (Fig. 4) to eliminate damaged mitochondria, and later activated mitochondrial biogenesis (Fig. 5) to increase mitochondrial membrane potential (Fig. 2c). To examine the long-term effects of Opti-ELF-

WMF, we exposed wild-type mice to Opti-ELF-WMF for 4 weeks, and observed increased mitochondrial membrane potential in the mouse liver by ~40% (Fig. 1b). Mitochondrial ETC complex II is comprised of four subunits, and Opti-ELF-WMF suppressed the activities of all the four subunits. The optimal conditions for ELF-WMF exposure to suppress the mitochondrial ETC activities were 1–8 Hz serial pulses for every 1 s, 10 μT magnetic field, and 4 ms pulse width, which are referred to as Opti-ELF-WMF in this communication. We previously reported that Opti-ELF-WMF most efficiently decreased the hysteresis of the electronic resistance of modified Ringer's solution as a function of temperature[20,21]. We have shown the effects of Opti-ELF-WMF on multiple cultured cells and wild-type mice for the first time. We found that the conditions of Opti-ELF-WMF exhibited the maximum effect on the reduction in the mitochondrial mass (Supplementary Fig. 2). The modifications of the pulse widths (Supplementary Fig. 2b) and the frequency profiles (Supplementary Fig. 2c) markedly attenuated the effects of ELF-WMF on the mitochondrial mass. As the conditions of Opti-ELF-WMF exerted the maximum effects on the hysteresis of electronic resistance of modified Ringer's solution in vitro and on the reduction of mitochondrial mass in vivo, the identity of the molecular target of Opti-ELF-WMF on ETC complex II subunits may share a feature similar to that of modified Ringer's solution.

The effects of magnetic fields on cultured cells, animal models, and humans have been reported mostly using static magnetic fields (SMFs) and radio frequency magnetic fields (RF-MF)[6,23,24]. Similarly, the effects of ELF-MF with static frequencies have also been reported[6,25], but the effect of ELF-MF with time-varying frequencies has not been reported. In addition, the intensities of SMF, RF-MF, and ELF-MF were mostly greater than 1 mT and were rarely less than 100 μT. In contrast, Opti-ELF-WMF had an MF intensity of 10 μT. According to the guidelines for limiting exposure to time-varying electric and magnetic fields by the International Commission on Non-Ionizing Radiation Protection (ICNIRP)[26], the intensities of time-varying MF acceptable for occupational exposure increase with decreasing frequencies. For example, MF intensities of 1 mT and lower are safe at less than 300 Hz. Adverse effects with higher MF intensities include induction of magnetic phosphenes by 5 mT ELF-MFs at 20 Hz[27]; gross external, visceral, or skeletal malformations by 20 mT LF-MF[28,29]; and genotoxicity to cells by 50 mT LF-MF[30]. Because beneficial

biological effects are sometimes inevitably accompanied by adverse effects, it is reasonable that biological effects of all the modalities of MF have been studied mostly with 1 mT or higher intensities.

Previous studies have shown that SMF, RF-MF, and ELF-MF increase, decrease, or have no effect on the levels of ROS in cultured cells and animal models[6]. In an SMF study, 200 μT decreased and 500 μT increased a surrogate marker of ROS, which suggested MF intensity-dependent changes in the levels of ROS[31]. We showed that ELF-WMF decreased the mitochondrial ROS level to 81% at 1 h and increased it to 114% at 12 h (Fig. 2c). The inconsistent effects of MF on the levels of ROS in previous reports may be partly accounted for by the temporal profiles of ROS levels.

We demonstrate that the target of Opti-ELF-WMF is the mitochondrial ETC complex II (Fig. 6a, b), but the underlying molecular mechanisms remain to be elucidated. Two models are proposed for the effect of WMF: the ion cyclotron resonance (ICR) effect as the classical mechanism[32,33] and the radical pair model as the quantum mechanism[34,35]. The radical pair model has been applied to the magnetic effect for cryptochrome (Cry). A magnetoreceptor protein (MagR) bound with Cry is also identified, which conducts a nanoscale magnetoreception in many organisms including mammals[36]. The Cry/MagR complex serves as a biocompass in these animals. The mitochondrial ETC complex II and Cry/MagR complex share the same components: flavin adenine dinucleotide (FAD) and iron–sulfur clusters. As mitochondrial ETC complexes I, II, and III have 8, 3, and 1 iron–sulfur clusters, respectively, FAD alone or a combination of FAD and iron–sulfur clusters may account for the effects of ELF-WMF. A moiety in the mitochondrial ETC complex II that is targeted by Opti-ELF-WMF may reside in a structure shared with the Cry/MagR complex.

We show that Opti-ELF-WMF induced mitophagy, followed by upregulation of the mitochondrial ETC activity. Similar to our observation, chemical inhibition of mitochondrial ETC complex II potentially provides neuroprotection by inducing autophagy in cultured neuronal cells[37]. Opti-ELF-WMF may be applicable to human diseases, in which amelioration of compromised mitophagy and enhancement of normal mitophagy would be beneficial.

Repetitive transcranial magnetic stimulation (rTMS) is approved by FDA for treating depression, migraine, and compulsive disorder. Similarly, according to the guidelines of evidence-based medicine for rTMS, level A evidence indicating definite efficacy is reached for depression and stroke-associated motor deficits[38,39]. In addition, level B evidence indicating probable efficacy is reached for Parkinson's disease, multiple sclerosis, fibromyalgia, aphasia, and post-traumatic stress disorder. Although the therapeutc mechanisms of rTMS remain mostly elusive, rTMS preserves mitochondrial membrane integrity in a rat model of ischemic stroke[40] and decreases oxidative stress in a rat model of autoimmune encephalomyelitis[41]. If rTMS and Opti-ELF-WMF share similar mechanisms, Opti-ELF-WMF may serve as a safe alternative to rTMS, in which the magnetic intensities up to 3.0 T potentially induce epilepsy and distressing sensation by stimulating the nerve and muscle[42,43].

We demonstrated that Opti-ELF-WMF induced mitophagy by inhibiting the mitochondrial ETC complex II activity, which was followed by hormetic facilitation of the mitochondrial ETC activity. We evaluated the effects under continuous exposure to Opti-ELF-WMF. However, discontinuation of the exposure or intermittent exposure might have exerted more effects. The temporal profile of Opti-ELF-WMF might be able to be optimized in the future.

Methods

The ELF-WMF device

The ELF-WMF device was manufactured by Mr. Kota Okada at the Technical Center of Nagoya University, Japan. The device had a round coil (1 cm height, 10.5 cm inner diameter, and 10.7 cm outer diameter, 50 turns of copper wire of 0.29 mm diameter). The current controller could generate the pulse width from 1 to 16 ms, magnetic flux intensity from 0 to 300 μT, and the pulse frequency from 1 to 16 Hz. We used a 10 μT magnetic field of 4 ms pulse width with increasing frequencies of 1, 2, 3, 4, 5, 6, 7, and 8 Hz in 8 s (Opti-ELF-WMF), unless indicated otherwise. This condition minimizes the hysteresis of the electronic resistance of modifid Ringer's solution[20,21]. Before and after each experiment, we confirmed the intensity of the magnetic flux using a pulse magnetic field meter (Aichi Micro Intelligent). To reduce the effects of electromagnetic fields generated by an incubator that had small motors at the

top and the bottom of the device, as well as by the geomagnetic field, two 5-mm thick copper plates were placed above and below the culture dish in a humidified incubator with 5% CO_2 at 37 °C (Supplementary Fig. 1a). The culture dish was placed directly on the coil, and the coil was placed 4 cm above the bottom copper plate. The two copper plates reduced the SMF at the culture dish in the incubator from 28 to 14 μT. Control cells were prepared simultaneously with ELF-WMF-stimulated cells from a single batch of cultured cells, and were analyzed in parallel in an identical setup to ELF-WMF-stimulated cells in another incubator of the same model, but with the ELF-WMF stimulator turned off. We confirmed that the application of the ELF-WMF stimulus of 300 μT for 24 h did not change the temperature of the culture medium in a 10 cm culture dish placed above the coil by a digital thermometer at 0.1 °C resolution (SN3000, Netsuken).

Exposure of wild-type mice to Opti-ELF-WMF

All the studies on mice were approved by the Animal Care and Use Committee of Nagoya University, and were conducted in accordance with the relevant guidelines. Seven-week-old C57BL6/N male mice were purchased from Japan SLC. Two Opti-ELF-WMF devices were placed in tandem beneath the mouse cage (Supplementary Fig. 1c). The intensities of magnetic fields, to which the mouse body was exposed, were from 5.3 to 14.2 μT above the coil (Supplementary Fig. 1d). Four to five mice were housed in a single cage for 4 weeks, and a total of 14 mice were analyzed for each of the Opti-ELF-WMF and control groups. For the control group, the cage was placed above the Opti-ELF-WMF device, but the switch was turned off. To evaluate the effect of Opti-ELF-WMF in a conventional environment, we did not use 5 mm thick copper plates in the mouse study.

Test for open-field locomotor activity in mice

Open-field locomotor activity was evaluated using a photometric actimeter (45 cm × 45 cm, IR Actimeter, Panlab). Fast and slow movements were monitored with a grid of infrared beams every 30 min for 24 h and were used as indices for locomotor activity. Eight mice were individually acclimated to the open-field locomotor activity test for 24 h. The mice were divided into two groups so that the average locomotive activities became similar between the

two groups. To examine the effects of Opti-ELF-WMF on the locomotor activity in mice, fast and slow movements were measured before (week 0) and after (week 4) exposure. All data were collected using the SEDACOM software (Panlab). Each mouse was tested individually and had no contact with other mice.

Isolation of mitochondria from the mouse liver

For the TMRM assay and the measurement of basal OCR, fresh mitochondria isolated from the mouse liver were examined, as described previously[44]. Briefly, a piece of liver was rinsed, minced, and disrupted in a mitochondrial isolation buffer (70 mM sucrose [Wako], 210 mM mannitol [Sigma], 5 mM HEPES [Dojindo], 1 mM EGTA [Sigma], and 0.5% [w/v] fatty acid-free BSA [Sigma], pH7.2) using a homogenizer. The homogenate was centrifuged at 800 × *g* for 10 min at 4 °C. The supernatant was then centrifuged at 8000 × *g* for 10 min at 4 °C. The pellet was suspended in the mitochondrial isolation buffer to obtain the mitochondrial fraction.

TMRM assay of the isolated mitochondria

The mitochondrial membrane potential of isolated mitochondria was analyzed using Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM) (T668, Thermo Scientific), following the procedure using a flow cytometer as described previously[45]. Briefly, the fresh isolated mitochondria were incubated with 100 nM TMRM in the mitochondrial isolation buffer for 30 min at 37 °C in a humidified incubator. The signal intensities of TMRM were quantified by FACSCalibur Flow Cytometer (BD Biosciences). Data were analyzed with CellQuest Pro (BD Biosciences).

Measurement of basal OCR of the isolated mitochondria

The basal OCR of the isolated mitochondria (20 µg of mitochondrial proteins per well) isolated from the mouse liver was determined using the Seahorse XFp Extracellular Flux Analyzer (Agilent Technologies). The assay was conducted as described previously[46].

Cell culture

Mouse hepatocyte-derived AML12 cell line was purchased from ATCC and cultured in DMEM/F-12 medium (Gibco) with 10% fetal bovine serum (FBS, Thermo Scientific), dexamethasone (Wako), and insulin-transferrin-sodium selenite (Sigma). HeLa, HEK293, Neuro2a, and C2C12 cells were also purchased from ATCC, and were cultured in DMEM (Gibco) with 10% FBS. PINK1 KO HeLa cells were kindly provided by Dr. Richard J. Youle from the National Institute of Neurological Disorders and Stroke[22], and were cultured in DMEM (Gibco) with 10% FBS. Human iPS cells were kindly provided by Dr. Toshiyuki Araki at the National Center of Neurology and Psychiatry, Japan, and were cultured on a plate coated with iMatrix-511 (892011, Takara Bio) in StemFit (AK02N, Ajinomoto) containing 125 ng/ml puromycin and 10 μM Y-27632 (030-24021, Wako Chemical). All cells were at ~60% confluency at 0 h of the Opti-ELF-WMF exposure, and did not reach 100% confluency in 24 h.

MitoSOX, MitoTracker Green, and TMRM assays of cultured cells

AML12 cells exposed to Opti-ELF-WMF for the indicated time periods were washed with PBS. MitoSOX (M36008, Thermo Scientific) and MitoTracker Green (M7514, Thermo Scientific) were dissolved in Hank's balanced salt solution (HBSS, Gibco) at 5 μM and 50 nM, respectively. TMRM was dissolved in the medium at 200 nM. Each dye was added to the cells and incubated for 30 min at 37 °C in a humidified incubator. The cells were then washed with PBS, trypsinized, resuspended in PBS, and harvested. Signal intensities of MitoSOX, MitoTracker Green, and TMRM were quantified by FACSCalibur Flow Cytometer (BD Biosciences) according to the manufacturer's protocols. Data were analyzed with CellQuest Pro (BD Biosciences).

Western blot analysis of cell lysates

Cultured cells or minced frozen mouse liver were lysed in PLC buffer containing 50 mM HEPES (pH 7.0), 150 mM NaCl, 10% glycerol, 1% TritonX-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 μg/μl aprotinin, 1 μg/μl leupeptin, 1 μg/μl pepstatin A, and 1 mM PMSF. The cell lysates were rotated at 4 °C for 20 min and centrifuged at 17,900 × *g* at 4 °C for 15 min. The supernatant was incubated at 37 °C for 1 h to analyze the mitochondrial ETC complex proteins or at 95 °C for 5 min to analyze other

proteins in the sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.005% bromophenol blue, and 2% 2-mercaptoethanol). For LC3-II, the lysates were separated by Tricine-SDS-PAGE on a 16% polyacrylamide gel[47]. For the other proteins, the lysates were separated by Tris-SDS-PAGE on a 10, 12, or 14% SDS-polyacrylamide gel. The samples were then transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore). Membranes were washed in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and blocked for 1 h at 24 °C in TBS-T with 5% skimmed milk. The membranes were then incubated overnight at 4 °C with specific antibodies, as indicated below. The membranes were washed with TBS-T and incubated with secondary anti-goat IgG (1:2000, sc-2094, Santa Cruz), anti-mouse IgG (1: 2000, LNA931V/AG, GE Healthcare), or anti-rabbit IgG (1: 2000, LNA934V/AE, GE Healthcare) antibody conjugated to horseradish peroxidase (HRP) for 1 h at 24 °C. Immunoreactive signals were detected with the ECL Western blotting detection reagents (GE Healthcare) and visualized using LAS 4000mini (GE Healthcare). Signal intensities were quantified using ImageQuant (GE Healthcare).

Antibodies for Western blot analysis

The following specific antibodies were used for Western blot analysis: anti-UQCRFS1 (1:1000 dilution, ab14746, Abcam), anti-NDUFS1 (1:1000, ab169540, Abcam), anti-VDAC1 (1:3000, ab14734, Abcam), OXPHOS cocktail (1:1000, ab110413, Abcam), anti-LC3 (1:1000, ab51520, Abcam), anti-PINK1 (1:500, ab23707, Abcam), anti-parkin (1:500, #4211, Cell Signaling Technology), anti-ubiquitin (1:1000, P4D1, BioLegend), anti-PGC-1α (1:1000, ab106814, Abcam), anti-PPARα (1:1000, GTX101098, GeneTex), anti-TFAM (1:1000, GTX103231, GeneTex), anti-ATP5A (1:1000, ab14748, Abcam), anti-SDHA (1:1000, GTX101689, GeneTex), anti-SDHB (1:2000, GTX104628, GeneTex), anti-SDHC (1:500, ab155999, Abcam), and anti-SDHD (1:500, ab189945, Abcam) antibodies.

Preparation of mitochondrial and cytosolic fractions of cultured cells

A mitochondria isolation kit (ab110170, Abcam) was used for the extraction of mitochondrial and cytosolic fractions according to the manufacturer's protocols. After obtaining the mitochondrial fraction by centrifugation at

12,000 × *g* for 10 min at 4 °C, the supernatant was used as the cytosolic fraction.

Detection of mitophagy in cultured cells

To detect mitophagy in AML12 cells, the Mitophagy Detection Kit (Dojindo Molecular Technologies) was used according to the manufacturer's protocol. Briefly, Mtphagy Dye (Dojindo) dissolved in HBSS at 100 nM was added to the cells and incubated for 30 min at 37 °C in a humidified incubator. After incorporation of Mtphagy Dye into AML12 cells, the cells were exposed to Opti-ELF-WMF for 120, 150, and 180 min, washed with PBS, trypsinized, resuspended in PBS, and harvested. Signal intensities of Mtphagy Dye were quantified by BD FACSCalibur Flow Cytometer (BD Biosciences) according to the manufacturer's protocols. Data were analyzed with CellQuest Pro (BD Biosciences). To visualize both mitophagy and lysosomes after exposure to Opti-ELF-WMF, Lyso Dye (Dojindo) dissolved in HBSS at 1 μM was also added to the cells and incubated for 30 min at 37 °C in a humidified incubator. Images were obtained using a confocal microscope TiE-A1R (Nikon).

Enzyme assay for mitochondrial ETC complex (I, II, III, and IV) activities of the mouse liver homogenates

Mitochondrial ETC complex activities were measured using homogenates of the frozen liver excised from C57BL/6N mice. The ETC complex activity assay was performed as previously described[48]. Briefly, the protein concentration of each sample was measured using a Pierce 660 nm protein assay reagent. The ETC complex activities of complexes I, II, III, and IV were estimated by determining the rotenone-sensitive decrease in absorbance of NADH at 340 nm, the decrease in absorbance of 2, 6-dichlorophenolindophenol (DCPIP) at 600 nm, the increase in absorbance of reduced cytochrome *c* at 550 nm, and the decrease in absorbance of reduced cytochrome *c* at 550 nm, respectively, with NanoDrop ONE^{C} (Thermo Scientific). The ETC complex activities were measured in the liver homogenate of mice that were exposed to either Opti-ELF-WMF or control in vivo. Similarly, the ETC complex activities were measured in the mouse liver homogenates before and after exposure to Opti-ELF-WMF for 8 min in vitro.

Enzyme assay for mitochondrial ETC complex II activity of the mitochondria isolated from the mouse liver

Mitochondria (10 µg protein) isolated from the liver of C57BL/6 N mice were used to measure the mitochondrial ETC complex II activities (Fig. 6e). Assays for measuring the fractional and extended mitochondrial ETC complex II activities of SQR, SCR, and SDH were performed as previously described[48,49]. The mitochondrial ETC complex II activities were quantified before and after exposure to Opti-ELF-WMF for 8 min *in cellulo*. The SQR, SCR, and SDH activities were measured by determining the decrease in absorbance of DCPIP at 600 nm, the increase in absorbance of reduced cytochrome *c* at 550 nm, and the decrease in absorbance of DCPIP at 600 nm, respectively, using NanoDrop ONE^{c} (Thermo Scientific). The SDHA activity was quantified by modifying a method used to measure the SDH (SDHA and SDHB) activity. For measuring the SDHA activity, 10 µg of sonicated mitochondrial fraction was resuspended in 35 mM phosphate buffer (pH 7.3) supplemented with 0.3 mM KCN (Wako), 10 µg/ml antimycin A (Sigma), 4 mM succinate (Wako), 1.6 mM phenazine methosulfate (PMS) (Sigma), and 40 µM DCPIP (Sigma). The SDHA activity was quantified before and after exposure to Opti-ELF-WMF for 8 min by determining the decrease in absorbance of DCPIP at 600 nm with NanoDrop ONE^{c} (Thermo Scientific).

Measurement of mitochondrial mass in AML12 cells exposed to an inhibitor of mitochondrial ETC complex I or II

AML12 cells were cultured either with variable concentrations of rotenone (Tokyo Chemical Industry Co.), an inhibitor of mitochondrial ETC complex I, or 3-nitropropionic acid (Cayman Chemical), an inhibitor of mitochondrial ETC complex II, for 12 h. The cells were then exposed to Opti-ELF-WMF for 3 h. Mitochondrial mass was measured by MitoTracker Green, as described above.

RNA-sequencing and GSEA of AML12 cells

Total RNA was extracted from AML12 cells exposed to OPTI-ELF-WMF for 1 h using QuickGene-Mini80 (Kurabo) according to the manufacturer's instructions. The extracted RNA was subjected to RNA-seq at Macrogen, Japan. Briefly, a sequencing library was prepared using the TruSeq Stranded mRNA kit (Illumina), and the library was read on an Illumina NovaSeq 6000

(150 bp paired-end reads). GSEA was conducted with the GSEA v4.1.0 software for Windows (https://www.gsea-msigdb.org/gsea/downloads.jsp) using the RNA-seq dataset. RNA-seq data were deposited in the gene expression omnibus (GEO) with an accession number GSE166811.

Statistics and reproducibility

All values are presented as the mean ± SD. Statistical significance was estimated either by *p*-value by Student's *t*-test, *p*-value by one-way ANOVA followed by Dunnett's posthoc test, or false discovery rate (*q*-value) of multiple Student's *t*-tests. *P*-values and *q*-values less than 0.05 were considered statistically significant. The numbers of replicates are indicated in each figure legend.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Additional data related to this paper are available upon request to the authors. RNA-seq data were deposited in the Gene Expression Omnibus with an accession number GSE166811. The original, uncropped blot images can be found in Supplementary Fig. 6. Source data can be found in the Supplementary Data.

Human Cell Diagram, Parts, Pictures, Structure and Functions

The cell is the basic functional in a human meaning that it is a self-contained and fully operational living entity. Humans are multicellular organisms with various different types of cells that work together to sustain life. Other non-cellular components in the body include water, macronutrients (carbohydrates, proteins, lipids), micronutrients (vitamins, minerals) and electrolytes. A collection of cells that function together to perform the same activity is known as tissue. Masses of tissue work collectively to form an organ that performs specific functions in the body. Despite this structural organization, all activity boils down to the cell – a complex unit that makes life possible.

Parts of the Human Cell

The cell contains various structural components to allow it to maintain life which are known as **organelles**. All the organelles are suspended within a gelatinous matrix, the **cytoplasm**, which is contained within the cell membrane. One of the few cells in the human body that lacks almost all organelles are the **red blood cells**.

The main organelles are as follows :

- cell membrane
- endoplasmic reticulum
- Golgi apparatus
- lysosomes
- mitochondria
- nucleus
- perioxisomes
- microfilaments and microtubules

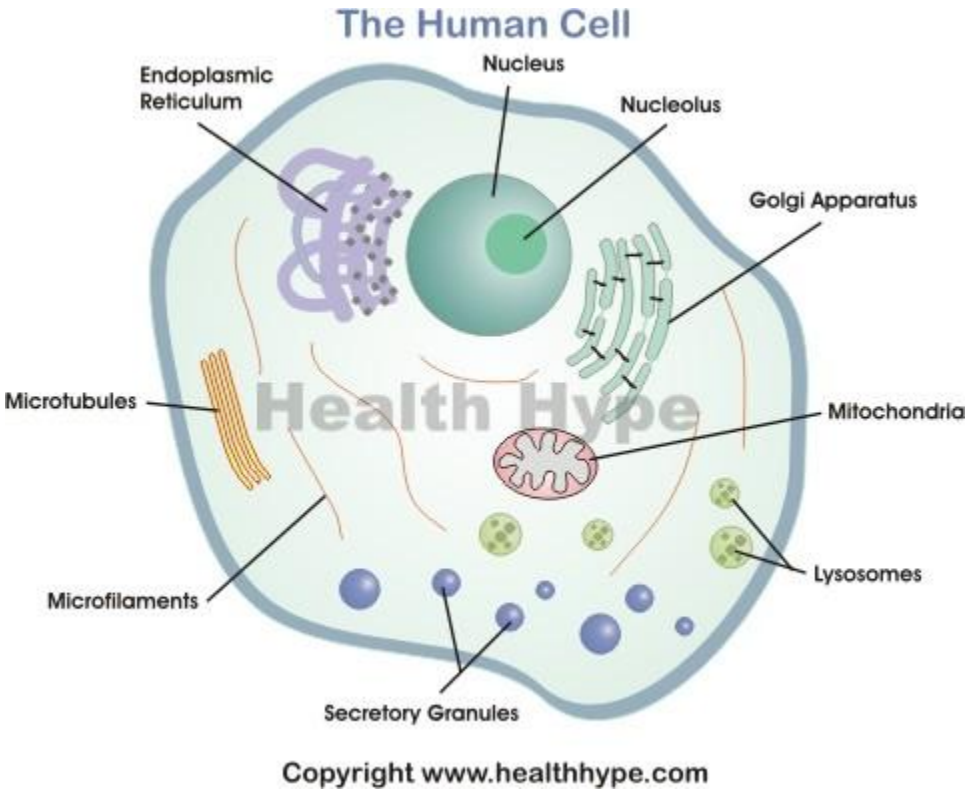


Diagram of the human cell illustrating the different parts of the cell.

Cell Membrane

The **cell membrane** is the outer coating of the cell and contains the cytoplasm, substances within it and the organelle. It is a double-layered membrane composed of proteins and lipids. The lipid molecules on the outer and inner part (lipid bilayer) allow it to selectively transport substances in and out of the cell.

Endoplasmic Reticulum

The **endoplasmic reticulum** (ER) is a membranous structure that contains a network of tubules and vesicles. Its structure is such that substances can move through it and be kept in isolation from the rest of the cell until the manufacturing processes conducted within are completed. There are two types of endoplasmic reticulum – rough (granular) and smooth (agranular).

- The **rough endoplasmic reticulum** (RER / granular ER) contains a combination of proteins and enzymes. These parts of the endoplasmic reticulum contain a number of ribosomes giving it a rough appearance. Its function is to synthesize new proteins.
- The **smooth endoplasmic reticulum** (SER / agranular ER) does not have any attached ribosomes. Its function is to synthesize different types of lipids (fats). The smooth ER also plays a role in carbohydrate and drug metabolism.

Golgi Apparatus

The **Golgi apparatus** is a stacked collection of flat vesicles. It is closely associated with the endoplasmic reticulum in that substances produced in the ER are transported as vesicles and fuses with the Golgi apparatus. In this way, the products from the ER are stored in the Golgi apparatus and converted into different substances that are necessary for the cell's various functions.

Lysosomes

Lysosomes are vesicles that break off from the Golgi apparatus. It varies in size and function depending on the type of cell. Lysosomes contain enzymes that help with the digestion of nutrients in the cell and break down any cellular debris or invading microorganisms like bacteria.

A structure that is similar to a lysosome is the **secretory vesicle**. It contains enzymes that are not used within the cell but emptied outside of the cell, for example the secretory vesicles of the pancreatic acinar cell release **digestive enzymes** which help with the digestion of nutrients in the gut.

Perioxisomes

These organelles are very similar to the lysosomes and contain enzymes that act together in the form of hydrogen peroxide to neutralize substances that may be toxic to the cell. **Perioxisomes** are formed directly from the endoplasmic reticulum rather than from the Golgi apparatus like lysosomes.

Mitochondria

These are the powerhouses of the cell and break down nutrients to yield energy. Apart from producing its own energy, it also produces a high-energy compound called ATP (adenosine triphosphate) which can be used as a simple energy source elsewhere. **Mitochondria** are composed of two membranous layers – an outer membrane that surrounds the structure and an inner membrane that provides the physical sites of energy production. The inner membrane has many infoldings that form shelves where enzymes attach and oxidize nutrients. The mitochondria also contain DNA which allows it to replicate where and when necessary.

Nucleus

The **nucleus** is the master control of the cell. It contains genes, collections of DNA, which determines every aspect of human anatomy and physiology. The DNA which is arranged into chromosomes also contains the blueprint specific for each type of cell which allows for replication of the cell. Within the nucleus is an area known as the **nucleolus**. It is not enclosed by a membrane but is just an accumulation of RNA and proteins within the nucleus. The nucleolus is the site where the ribosomal RNA is transcribed from DNA and assembled.

Microfilaments and Microtubules

Microfilaments and **microtubules** are rigid protein substances that form the internal skeleton of the cell known as the **cytoskeleton**. Some of these microtubules also make up the **centrioles** and **mitotic spindles** within the cell which are responsible for the division of the cytoplasm when the cell divides. The microtubules are the central component of **cilia**, small hair-like projections that protrude from the surface of certain cells. It is also the central component of specialized cilia like the tail of the sperm cells which beats in a manner to allow the cell to move in a fluid medium.

Functions of the Human Cell

The functions of the human cell varies based on the type of cell and its location in the human body. All the organelles work together to keep the cell alive and allow it to carry out its specific function. Sometimes these organelles are highly specialized and can vary in size, shape and number. The organelles are the most basic functional units but it cannot exist and operate without the cell as a whole. Its functions include intake of nutrients and other substances, processing of these compounds, production of new substances, cell replication and energy production. In specialized cells that need to be motile, like sperm cells, tail-like projections allow for cellular locomotion. The function of each organelle has already been discussed but is worth considering in summary.

- The cell membrane allows substances to enter and leave the cell. While certain substance like oxygen can easily diffuse through the cell membrane, others have to actively transported through the process of endocytosis. Small particles are transported by the process of pinocytosis while larger particles are moved by the process of phagocytosis. These functions can become highly specialized to allow cells to perform specific activities, like the macrophages that phagocytose invading bacteria to neutralize it.
- Small and large substances that do not dissolve in the cytoplasm are contained within vesicles. Lysosomes attach to the vesicles and digest this material.
- The endoplasmic reticulum and Golgi apparatus synthesize different substances like protein and fats as required by the cell or designated according to its specific function. It utilizes basic nutrient molecules that are either dissolved in the cytoplasm or specific substances contained within vesicles.
- Some nutrients, specifically carbohydrates, are transported to the mitochondria where it is broken down further to yield energy. In the process, high-energy molecules known as ATP (adenosine triphosphate) are manufactured and provide energy for other organelles.
- The genetic material housed in the nucleus provides the blueprint necessary for the production of specific compounds by the endoplasmic reticulum and Golgi apparatus. The genes also help the cell replicate and codes for the formation of new cells.
- Secretory vesicles store some of the enzymes and other specialized substances formed by the endoplasmic reticulum and Golgi apparatus. These stored substances are released from the cell when necessary in order to complete various functions that allow the body to function as a whole.

Turning back time with emerging rejuvenation strategies

Salah Mahmoudi [1,4], Lucy Xu [1,2,4], Anne Brunet [1,3,*]

PMCID: PMC7653017 NIHMSID: NIHMS1638129 PMID: 30602763
The publisher's version of this article is available at Nat Cell Biol

Abstract

Ageing is associated with the functional decline of all tissues and a striking increase in many diseases. Although ageing has long been considered a one-way street, strategies to delay and potentially even reverse the ageing process have recently been developed. Here, we review four emerging rejuvenation strategies—systemic factors, metabolic manipulations, senescent cell ablation and cellular reprogramming—and discuss their mechanisms of action, cellular targets, potential trade-offs and application to human ageing.

Ageing represents a major risk factor for many chronic conditions, including cardiovascular disease, diabetes, cancer, arthritis and frailty[1,2]. Once considered irreversible, ageing is in fact remarkably malleable. Indeed, inhibition of high-nutrient-sensing pathways (for example, the insulin–insulin-like growth factor (IGF) and mechanistic target of rapamycin (mTOR) pathways) and activation of low-nutrient-sensing proteins (for example, 5′ AMP-activated protein kinase (AMPK) and sirtuins) extend lifespan in various model organisms[3,4]. Diet-based interventions, such as dietary restriction, and pharmacological interventions, including the mTOR inhibitor rapamycin, improve aspects of ageing even when administered late in life[5-10]. A key question is whether ageing of cells, tissues and organisms can be reversed or ‘rejuvenated’ rather than simply delayed.

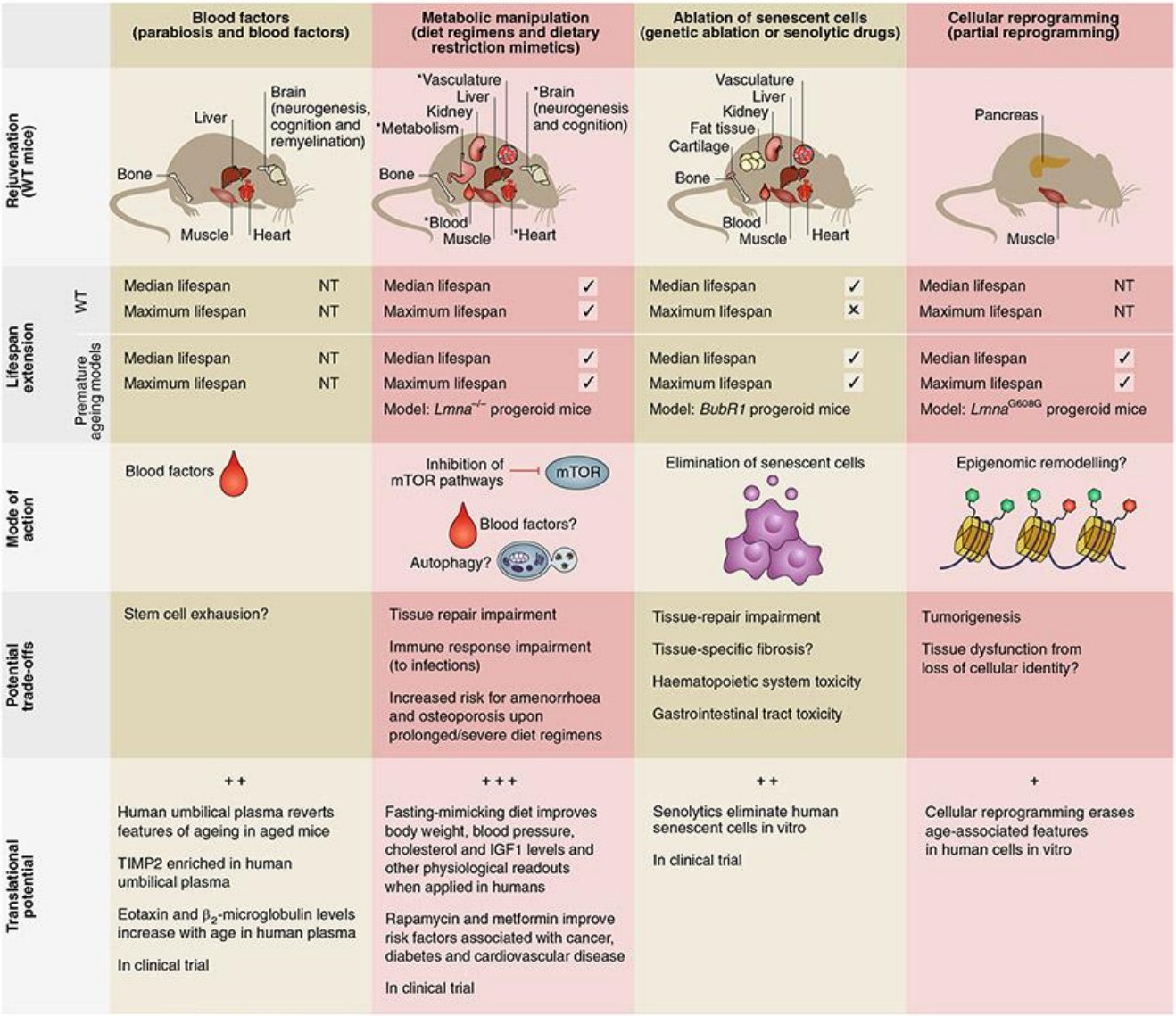
A host of age-associated features have been identified, with a subset being potential drivers of the ageing process (extensively reviewed elsewhere[1,2]). At the molecular level, ageing hallmarks comprise DNA damage, epigenetic alterations, telomere attrition, protein aggregation and accumulation of aberrant mitochondria and lysosomes[1,2]. At the cellular and organismal level, ageing features include cellular senescence, stem cell exhaustion, deregulated nutrient sensing and chronic low-grade inflammation[1,2]. Various rejuvenation strategies that target these hallmarks have recently emerged and they fall into four broad categories: systemic (blood) factors, metabolic manipulations, senescent cell ablation and cellular reprogramming. Although these approaches seemingly target very different ageing features[11-15], a central question is whether they share common mechanisms of action. This Review discusses these four rejuvenation strategies and how they improve health and lifespan. We also address several key questions: which hallmarks of ageing are targeted by each strategy and are

there commonalities in their modes of action? Does the rejuvenating effect come with trade-offs? Ultimately, can rejuvenation strategies be used to improve human health and longevity and target age-associated diseases?

Blood factors as targets for rejuvenation

Heterochronic parabiosis studies, in which the circulatory systems of a young mouse and an aged mouse are fused, have provided compelling evidence that blood factors influence organismal ageing (Fig. 1, Table 1 and Supplementary Table 1). Heterochronic parabiosis was initially shown to revitalize muscle stem cells in naturally aged mice, reversing the age-dependent decline in stem cell activation and number and improving their age-associated differentiation bias[16,17]. Since then, heterochronic parabiosis has been shown to enhance muscle, liver, brain and heart function of aged mice[17-24], by boosting the function of both stem and differentiated cells[17-23]. Sharing blood circulation with a young mouse also reduces genomic instability in the aged mouse[20] and reverses age-associated gene expression signatures[25]. Blood factors, rather than blood cells, seem to play a major role in these rejuvenating effects[24-26]: direct injection of young blood plasma (devoid of cells)[25] or of human umbilical cord plasma (also devoid of cells)[26] into aged mice can recapitulate several aspects of heterochronic parabiosis, notably the increase in neurogenesis and improvement of cognitive functions[25,26] (Table 1 and Supplementary Table 1). These observations raise the possibility that blood factors (for example, proteins, metabolites, lipids and exosomes) could be used to reverse aspects of the ageing process, perhaps even in humans.

Fig. 1 |. Comparison of emerging strategies for organismal rejuvenation and lifespan.



A comparison of the four emerging rejuvenation strategies: blood factors, metabolic manipulation, ablation of senescent cells and cellular reprogramming. The figure depicts the features that improve when treatment in mice is initiated at midlife or later. The top panel shows organs or tissues that exhibit a rejuvenated phenotype in wild-type (WT) mice. For rapamycin, features that have been shown to improve also in young mice following treatment are indicated with an asterisk (*). The effect on lifespan, proposed primary mode (or modes) of action and possible trade-offs of these strategies are also presented. Finally, the translational potential in humans is indicated by the increasing number of plus signs (+) based on present evidence in human ageing and current feasibility. NT, not tested. Question marks indicate possible modes of action and trade-offs. Figure adapted from ref. [188].

Table 1 |.

Summary of studies testing rejuvenation interventions at midlife or later in naturally ageing mice

Intervention	Age at intervention (months)	Metric output	Comparison points (control)	Ref.
Blood factors				
Parabiosis	2–3, 19–26	Skeletal muscle (MuSC) and liver regeneration	Old-old and young-young parabionts	17
Parabiosis	4–6, 24–26	Muscle regeneration (MuSC and fibrosis)	Old-old and young-young parabionts	16
Parabiosis	3–4, 18–20	Neurogenesis and cognitive function	Old-old and young-young parabionts	21
Parabiosis	1–2, 10–12	Spinal cord remyelination	Middle-aged-middle-aged and young-young parabionts	24
Parabiosis	2, 23	Cardiac metrics	Old-old and young-young parabionts	18
Parabiosis	2, 15–16 or 21	Neurogenesis and cognitive function	Old-old and young-young parabionts	22
Parabiosis	2–3, 22–24	Muscle regeneration (MuSC) and function	Old-old and young-young parabionts	20
Parabiosis	3, 18	Synaptic plasticity and gene expression	Old-old parabionts	25
Parabiosis	3, 19	Bone regeneration	Old-old and young-young parabionts	19
Parabiosis	3, 18	Neurogenesis and cognitive function	Young-young parabionts	23
Young blood injection	18	Cognitive function and gene expression	Old blood	25
Human plasma injection (cord, young and elderly)	8–10, 13–14	Neuronal and cognitive functions and gene expression	Age-matched vehicle control, young (22 years of age) and old (66 years of age) human plasma	
TIMP2 administration	18	Synaptic plasticity and cognitive functions	Age-matched vehicle control	26
Oxytocin administration	2–4, 22–24	Muscle regeneration (MuSC and fibrosis)	Age-matched vehicle and antagonist (only young) control	36
GDF11 administration	23–24	Cardiac metrics	Age-matched vehicle control	18

Intervention	Age at intervention (months)	Metric output	Comparison points (control)	Ref.
GDF11 administration	21–23	Neurogenesis and cognitive function	Age-matched vehicle control	22
GDF11 administration	2–3, 22–24	Muscle regeneration (MuSC) and function	Age-matched vehicle control	20
GDF11 administration	23	Muscle regeneration (MuSC)	Age-matched vehicle control	34
GDF11 administration	24	Cardiac metrics and function	2 months of age, 3 months of age and age-matched vehicle treated	33
GDF11 administration	2, 22	Cardiac metrics and body weight	Age-matched vehicle control	32
Metabolic manipulation				
Short-term dietary restriction	5–8, 28–30	Vasculature metrics	Age-matched ad libitum	43
Short-term dietary restriction	2, 18	Skeletal muscle (MuSC)	Age-matched ad libitum	7
Fasting-mimicking diet	16	Organ size and regeneration	16 months of age and age-matched ad libitum	5
Fasting-mimicking diet	16	Immunosenescence	4 months of age, 16 months of age and age-matched ad libitum	5
Fasting-mimicking diet	16	Cognitive function	Age-matched ad libitum	5
Fasting-mimicking diet	16	Bone density	12 months of age and age-matched ad libitum	5
Fasting-mimicking diet	16	Cancer and inflammation	Age-matched ad libitum	5
Ketogenic diet	12	Physiological and metabolic metrics; physical, behaviour and cognitive functions	Age-matched ad libitum and low-carbohydrate non-ketogenic	
Ketogenic diet	12–14	Cognitive and motor function and frailty index	12 months of age and age-matched ad libitum	8
Ketogenic diet	12–14	Cognitive and motor function	12 months of age and age-matched ad libitum	8
Rapamycin	22	Immune system (HSC and immunity)	2 months of age and age-matched vehicle control	48

Intervention	Age at intervention (months)	Metric output	Comparison points (control)	Ref.
Rapamycin	4, 13, 20–22	Comprehensive organismal assessment (>25 tissues)	3–6 months of age and age-matched vehicle control	49
Metformin	12	Serum biomarkers	Age-matched ad libitum and dietary restricted	41
Metformin	12	Physical performance	Age-matched ad libitum	41
Metformin	12	Liver, muscle and gene expression	Age-matched ad libitum and dietary restricted	41
Resveratrol	12	Physiological metrics and gene expression	Age-matched untreated controls	54
Resveratrol	18	Renal function and histology	Age-matched untreated control	53
Ablation of senescent cells				
Ablation of p16-positive cells	18	Adipose tissue metrics	Age-matched wild-type treated	88
Ablation of p16-positive cells	12	Kidney, heart and adipocyte metrics and function	12 months of age and age-matched vehicle control	62
Ablation of p16-positive cells	24	Vasculature function	Age-matched vehicle control	82
Ablation of p16-positive cells	12	Cartilage degeneration	Age-matched vehicle control	67
Ablation of p16-positive cells	24	Fat accumulation in liver	Age-matched vehicle control	64
Ablation of p16-positive cells	12, 20	Bone metrics and loss	Age-matched vehicle control	69
Ablation of p16-positive cells	>25	Renal function	Age-matched vehicle control	61
Dasatinib + quercetin	24	Cardiac metrics and function	Age-matched vehicle control	68
Dasatinib + quercetin	24	Vasculature function	Age-matched vehicle control	82
Dasatinib + quercetin	24	Fat accumulation in the liver	Age-matched vehicle control	64
Dasatinib + quercetin	20	Bone metrics and loss	Age-matched vehicle control	69
Dasatinib + quercetin	20	Physical performance	Age-matched vehicle control	70
ABT263	21–22	Immune system (HSC) and muscle (MuSC) function	2 months of age and age-matched vehicle control	65

Intervention	Age at intervention (months)	Metric output	Comparison points (control)	Ref.
UBX0101	2–3, 19–20	Cartilage regeneration	Age-matched vehicle control	67
FOXO4-DRI	24	Renal function and frailty	Age-matched vehicle control	61
Cellular reprogramming				
Transient reprogramming	12	Pancreas regeneration	Age-matched vehicle control	105
Transient reprogramming	12	Muscle regeneration	Age-matched vehicle control	105

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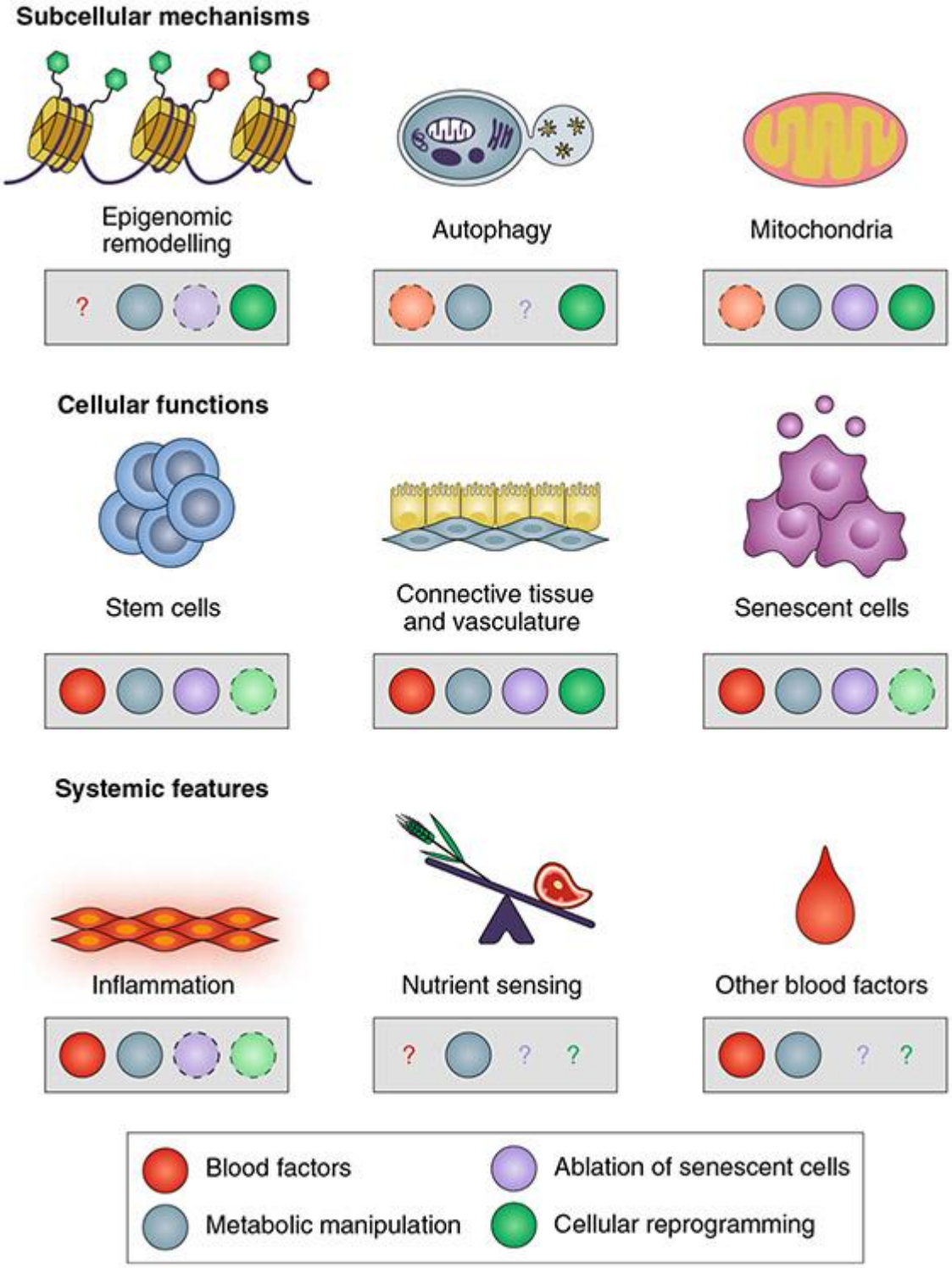
An extended version of this table with more details regarding the method of administration or procedure, duration of intervention, mouse strain and sex is available as Supplementary Table 1 HSC, haematopoietic stem cell; MuSC, muscle stem cell.

How does young blood revitalize aged organs and tissues? Young blood may contain pro-rejuvenation factors, or it could dilute or inhibit pro-ageing factors in aged blood (Fig. 2). The pro-rejuvenation effect of young blood on the liver, muscle and brain is less pronounced than the pro-ageing effect of aged blood on these tissues[27], suggesting the presence of potent pro-ageing factors in aged blood. Indeed, systemic pro-ageing factors have been identified through heterochronic parabiosis, including eotaxin (also known as CCL11)[21] and β_2-microglobulin[23]. The levels of eotaxin and β_2-microglobulin increase with age, and these factors inhibit neurogenesis and cognition in young mice[21,23]. Whether blocking pro-ageing blood factors improves tissue function in aged mice remains to be shown, but aged β_2-microglobulin knockout mice exhibit enhanced neurogenesis and cognitive functions compared to age-matched wild-type mice[21,23]. Other systemic signalling pathways have been implicated in mediating the pro-ageing effect of aged blood[16,17,28-31]. For example, heterochronic parabiosis reverses the excessive Wnt signalling underlying the differentiation bias of aged muscle stem cells[16,29]. Furthermore, systemic attenuation of transforming growth factor-β signalling improves age-dependent decline in neurogenesis and myogenesis[30], and inhibition of interferon signalling partially ameliorates neurogenesis and cognitive function in aged mice[31]. Thus, several pro-ageing factors have been identified in aged blood.

Fig. 2 |. Potential common mechanisms and target cells of the rejuvenation strategies.



A comparison of the proposed underlying mechanisms of action and target cell types influenced by each of the rejuvenation strategies. These include subcellular mechanisms (for example, chromatin changes, induction of autophagy pathways and alteration in mitochondrial function), cellular functions (such as revival of stem cell populations, attenuation of the deleterious effects of senescent cells and changes in connective tissue cells (for example, endothelial cells, fibroblasts and adipocytes)) and intercellular features (for example, decrease in inflammation, perturbation of nutrient-sensing pathways and changes in blood factors). The circles below each feature are colour-coded for each rejuvenation strategy and represent the current level of evidence for the effect of the corresponding strategy on the feature. Solid/dark circles, strong evidence. Dotted/light circles, mostly indirect evidence. Question marks, no evidence as of now.

Identifying rejuvenation factors in young blood has been more difficult. Heterochronic parabiosis can restore the decreased Notch signalling that underlies the decline in muscle stem cell activation and number[17,28], although the specific systemic factor (or factors) remains unclear. Growth/differentiation factor 11 (GDF11) was initially identified as a circulating factor whose levels decrease with age but are restored through heterochronic parabiosis[18]. Exogenous GDF11 can rejuvenate heart function[18,32] and improve muscle and neural stem cell functions in aged mice[20,22] (Table 1 and Supplementary Table 1). However, subsequent studies reported no beneficial effects of GDF11 on heart and muscle stem cell function[33,34], and injection of GDF11 can induce cachexia[35]. Thus, although GDF11 may have beneficial effects under specific conditions, it is unlikely to be a universal mediator of rejuvenation. Another potential rejuvenating blood factor whose levels decrease with age is the hormone oxytocin[36]. Its systemic administration improves muscle regeneration by enhancing muscle stem cell activation and/or proliferation in aged mice[36] (Table 1 and Supplementary Table 1). As oxytocin is known for its role in social bonding[37], it could potentially link social environment and ageing. Finally, TIMP2, a metalloproteinase inhibitor, was identified in human umbilical cord plasma[26] and its levels have been shown to decrease with age in both mice and humans[26]. Injection of human cord blood induces hippocampal neurogenesis and improves learning and memory in naturally aged mice[26], effects that are attenuated by TIMP2 depletion[26]. Moreover, administration of exogenous TIMP2 can improve cognitive function in aged mice, pointing to TIMP2 as a key rejuvenating factor[26] (Table 1 and Supplementary Table 1).

These studies suggest the presence of both pro-ageing and anti-ageing factors in the blood, which can be targeted to reverse age-related decline in multiple tissues. However, many open questions remain. Which cell types secrete these factors and could these cells be targeted to achieve similar effects? Do circulating factors drive rejuvenation of all tissues or do they have tissue-specific action? Comprehensive analysis of the response of multiple organs to blood factors will be required to address this question. Testing the interaction between individual factors, including pro-ageing and rejuvenation factors, could identify the main contributors and allow for combinatorial

treatments to revitalize tissues and organs. A central question is whether young blood or specific blood factors can extend organismal lifespan. Although initial studies using young blood in aged mice or GDF11 in progeroid mice reported little effect on overall lifespan[38,39], a thorough investigation of the effect of blood factors on mammalian lifespan will be important.

Metabolic-induced rejuvenation

Long-term dietary restriction extends healthspan and lifespan across several species[12,40]. Less-restrictive diet regimens and drugs that mimic the metabolic effects of dietary restriction also have beneficial effects on lifespan[5,8-10,41,42]. Until recently, it was unclear whether these interventions could reverse ageing features in aged individuals. Initial studies on short-term dietary restriction (5 days to 12 weeks) in middle-aged or old-aged mice revealed improved function in multiple tissues, including muscle, bone, liver, brain, vasculature and immune system[5,7,43] (Fig. 1, Table 1 and Supplementary Table 1), consistent with the possibility that dietary interventions could indeed reverse functional decline. Here, we focus on dietary interventions or mimics that are initiated at middle age or later and on their potential rejuvenation effects on ageing hallmarks.

The periodic fasting-mimicking diet (FMD) consists of cycles of very low caloric intake for 4 days, repeated twice per month, with ad libitum feeding in between[5]. When initiated in 16-month-old mice, FMD reverses age-associated haematopoietic differentiation bias, increases hippocampal neurogenesis and improves hippocampus-dependent memory[5] (Table 1 and Supplementary Table 1). FMD also increases median lifespan and decreases cancer incidence and inflammatory diseases, including ulcerative dermatitis[5]. Some of the beneficial effects of FMD are probably mediated by an increased proliferative capacity and number of stem cells[5]. The refeeding portion of FMD may play a key role in this, as it results in a boost in cell proliferation[5]. Whether FMD also improves tissue function by selecting against dysfunctional cells is unclear. The proliferation boost following refeeding may favour youthful cells, diluting out damaged ones and improving overall tissue function. Given that FMD (and the ketogenic diet, discussed below) reduces cancer incidence[5,9], such regimens may also select against cancerous or precancerous cells.

The ketogenic diet involves the same caloric intake as a normal diet but with reduced carbohydrate consumption. This diet mimics many of the metabolic changes occurring in mice under dietary restriction or fasting[8,9,44]. Both fasting and a ketogenic diet decrease blood glucose levels and increase ketone body levels and fatty acid oxidation[8,9,44] (Table 1 and Supplementary Table 1). Interestingly, alternating between a ketogenic and a control diet weekly in middle-aged mice improves recognition memory and midlife survival[8]. A non-cyclic ketogenic diet also increases median lifespan and improves motor function in aged mice while decreasing cancer incidence[9]. Although not explicitly stated in these studies, some measurements are similar or better post-

treatment compared to those at the time of treatment initiation, suggesting not only a delay but also a reversal of the measured features[9,10]. Thus, manipulating diet content may constitute an effective approach for reversing ageing hallmarks and may be easier to implement in humans than long-term dietary restriction.

How do these diet regimens rejuvenate aged tissues? Nutrient-sensing pathways, including mTOR and insulin–IGF signalling, could play a key role[3,45-47] (Fig. 2). Periodic FMD was proposed to act by reducing insulin–IGF1 signalling and inhibiting the activity of mTOR and protein kinase A[6]. Short-term treatment (6 weeks) with rapamycin, an mTOR inhibitor, improves haematopoietic stem cell function in aged mice (although not to the level of 2-month-old mice) and extends lifespan[48] (Table 1 and Supplementary Table 1). The insulin and mTOR signalling pathways are also known to regulate autophagy[3,45-47], and FMD can indeed counteract the decline in autophagy-related proteins in ageing muscle[5]. This points to an important role of mTOR in mediating the beneficial effects of these regimens and raises the possibility that mTOR inhibitors could be used to rejuvenate ageing tissues. Although the effect of rapamycin on lifespan is well established[45], whether it is a rejuvenating compound remains debated. A comprehensive assessment of ageing phenotypes following long-term (1 year) treatment of young and aged mice showed that rapamycin improves several features, including memory and learning[49]. However, it also ameliorates some of these features in young mice, suggesting that it may have age-independent positive effects[49].

Similarly to periodic FMD, the ketogenic diet also inhibits mTOR and insulin–IGF signalling[8,9]. Interestingly, although a short-term ketogenic diet (1 month) does affect the expression of genes related to insulin signalling and fatty acid synthesis, an extended ketogenic diet (14 months of cyclic diet) does not affect these genes[8]. Thus, repeated cycles may become less effective on signalling pathways[8]. Ketogenic effects could be mediated by increased circulating β-hydroxybutyrate levels, a ketone that inhibits histone deacetylases and may thereby link metabolism, epigenetics and rejuvenation[8,9]. Hence, β-hydroxybutyrate could represent an effective longevity and rejuvenating compound[50,51].

Other nutrient-sensing pathways could also be involved in the rejuvenation effects of dietary regimens (Table 1 and Supplementary Table 1). For example, metformin, which increases AMPK activity[42,47], preserves mitochondrial function and decreases inflammation when administered starting at middle age[41]. Resveratrol, which can activate sirtuins (and other nutrient-responsive pathways), also improves cognitive and renal function and reduces inflammation in rodents when initiated at mid-to-late life[42,52-54]. Whether these improvements represent a true reversal of pre-existing ageing phenotypes remains an open question.

Ablation of senescent cells to restore tissue youthfulness

Cellular senescence is a cell-intrinsic mechanism induced by stress that prevents propagation of damaged cells[55,56]. Initially identified as a barrier against tumour development[56], senescence is now known to be involved in tissue remodelling during embryogenesis[57,58], wound healing[59,60] and ageing[61-70]. Senescence markers include senescence-associated β-galactosidase activity, the cell-cycle inhibitors $p16^{INK4a}$ and $p21^{CIP1}$, and many secreted inflammatory factors (collectively referred to as the senescence-associated secretory phenotype (SASP))[55,56,71]. Senescent cells are heterogeneous[72,73] and do not always exhibit all markers, and, conversely, some markers are also present in non-senescent cells[71,74]. Senescent cells accumulate in ageing tissues across organisms, including primates and rodents, and in age-related pathologies, such as atherosclerosis and Alzheimer's disease[75-79]. Accordingly, senescence has long been thought to contribute to organismal ageing[56], although whether it is a cause or consequence is only starting to be resolved. Indeed, mouse models and compounds that trigger senescent cell elimination have revealed that targeting senescent cells can reverse or delay aspects of the ageing process[61-70] (Fig. 1).

The first evidence that senescent cells can actively contribute to ageing came from genetically modified mice that allow for inducible elimination of p16-positive cells in the context of a progeroid disease[63]. In INK-ATTAC transgenic mice that express a drug-inducible form of caspase 8 under the *Cdkn2a* (which encodes $P16^{Ink4a}$) promoter, drug administration triggers caspase-8-mediated apoptosis in p16-positive cells[63]. In a progeroid mouse model (*BubR1*), caspase-8-mediated ablation of p16-positive cells starting from early life delays the onset of age-associated features, including loss of fat and skeletal muscle and cataract development[63]. Even later in life, ablation of p16-positive cells reduces age-associated fat and skeletal muscle loss[63]. Follow-up studies in naturally ageing mice showed that the removal of p16-positive cells starting from 12 months of age (midlife) through to 18 months of age attenuates age-associated decline in adipocyte, kidney and heart function[62] (Table 1 and Supplementary Table 1). Importantly, the removal of p16-positive cells from midlife to end of life extends median lifespan by 24–27%[62]. Similar health benefits were observed with another model. In p16-3MR mice that express thymidine kinase from herpes simplex virus under the *Cdkn2a* promoter, administration of the thymidine kinase substrate ganciclovir initiates apoptosis of p16-positive cells[59]. In both mouse models, apoptosis induction in p16-positive cells late in life for at least 3 weeks improves liver, kidney, bone and adipocyte metrics and function[61,64,69]. Although not specifically stated, some metrics seem to be better after treatment than at initiation[61,64], suggesting that senescent cell ablation may reverse ageing features. However, high p16 expression has also been observed in non-senescent cells, notably macrophages[71,74]. Hence, the beneficial effects of this intervention might partially be due to targeting macrophages, which are known to change with age[1,2,80]. Despite the promise of these initial findings, more remains to be learned about the optimal times for treatment initiation and duration for maximal effects, and about specificity to senescent versus immune cells.

These initial proof-of-concept studies spurred the field to identify compounds that can relatively specifically kill senescent cells based on their unique molecular profiles. Several classes of such 'senolytic' drugs have been identified, including Bcl protein family inhibitors (for example, navitoclax, also known as ABT263)[65], kinase inhibitors (for example, dasatinib and quercetin)[68], heat shock protein 90 inhibitors (for example, 17-DMAG)[66] and inhibitors of the p53–MDM2 interaction (for example, UBX0101)[67,81]. Dasatinib and quercetin[68] and 17-DMAG[66] improve healthspan in the *Ercc1*$^{-/-}$ progeroid mouse model. In naturally aged mice, senolytics enhance cardiovascular, vascular, bone, liver and physical functions (dasatinib and quercetin)[64,68,69,82], revitalize haematopoietic and muscle stem cell populations (ABT263)[65], enhance cartilage regeneration (UBX0101)[67] and even extend median lifespan (dasatinib and quercetin)[70] (Table 1 and Supplementary Table 1). A forkhead box protein O4 peptide (FOXO4-DRI) also has senolytic effects. This peptide blocks the sequestration of p53 by FOXO4, which seems to be senescence specific, thus allowing p53 activation and cell death in senescent cells[61]. FOXO4-DRI restores fitness, fur density and kidney function in both progeroid (*Xpd*$^{TTD/TTD}$) and naturally aged mice[61] (Table 1 and Supplementary Table 1). Whether FOXO4-DRI acts on all types of senescent cells without targeting healthy cells, a common challenge for senolytic drugs[14,68,83-85], remains to be determined. Many senolytics were initially identified as cancer drugs because cancer cells exploit similar anti-apoptotic pathways, notably overexpression of Bcl family proteins[84,86]. Thus, some beneficial effects of senolytics may originate from the elimination of precancerous and cancerous cells.

How does the removal of senescent cells rejuvenate tissues and extend lifespan? Senescence could contribute to the decline in tissue homeostasis and function by inducing a permanent cell-cycle arrest in proliferative cell populations. Senescence of reparative stem and progenitor cells may lead to a decline in tissue regenerative potential. Senescence could also act through SASP, which promotes local and systemic inflammation[55,56]. SASP factors could contribute to stem cell exhaustion or dysfunction, infiltration and alteration of immune cells, insulin resistance, damage of tissue structure and even propagation of the senescent phenotype in neighbouring cells[56,87]. Elimination of senescent cells can revive stem cell populations in naturally aged mice[65,88] (Fig. 2), and p16 depletion resets ageing features in aged muscle stem cells[89]. Moreover, SASP inhibition by the Janus kinase 1/2 inhibitor ruxolitinib reduces systemic and adipose tissue inflammation and increases insulin sensitivity in naturally aged mice[69,88,90]. Senescent cell removal can delay cancer development, which could be a source of the observed lifespan extension in mice[62]. Elucidating the mechanisms by which senolytics ameliorate tissue function will be important in identifying additional senolytic compounds and in determining how best to use them. Importantly, senescent cells can have beneficial effects, for example, by facilitating tissue repair after injury and preventing tissue fibrosis[59,60,91]. Identifying mechanisms that distinguish between the beneficial and harmful effects of senescence could help to identify therapeutic strategies to specifically target the latter.

Reprogramming back to a youthful state

Cellular reprogramming is the conversion of terminally differentiated somatic cells into induced pluripotent stem cells (iPSCs)[92], for instance by the expression of the transcription factors OCT4 (also known as POU5F1), SOX2, KLF4 and MYC (OSKM)[92]. Cellular reprogramming allows for the generation of in vitro models to study ageing and age-associated diseases and the development of autologous stem cell therapies to replace ageing tissues[93-95]. Reprogramming also resembles to some extent the process of fertilization, during which the chronological age of the parent cells is effectively reset such that the resulting offspring has a normal lifespan[96]. Hence, cellular reprogramming has emerged as a potential rejuvenation strategy[15,96].

Reprogramming to pluripotency can erase several ageing features in vitro. iPSCs derived from aged cells show extended telomeres, improved mitochondrial morphology, number and fitness (ATP production and membrane potential) and restored nuclear morphology[15,95,97,98]. iPSC reprogramming of aged cells also resets heterochromatin marks and transcriptomic profiles[15,95,97,99]. After re-differentiation of these iPSCs into neurons or fibroblasts, transcriptomic changes, improvements in nucleocytoplasmic compartmentalization, nuclear morphology and (in the case of fibroblasts) proliferative potential largely remain in the rejuvenated state[95,97,99]. This suggests that the youthful state is not exclusive to pluripotency and can persist after re-differentiation. Although most age-associated phenotypes tested are reversed by in vitro reprogramming, iPSCs generated from aged human cells can retain a DNA methylation signature of their age, which can be erased with additional passaging[100]. Thus, some features of ageing may be harder to rejuvenate than others, and some aspects, such as pre-existing genetic mutations, cannot be reverted[94,100]. The ability to rejuvenate ageing traits may be specific to reprogramming to a pluripotent state because direct reprogramming to a differentiated state (for example, neurons) was less effective at erasing ageing marks[99]. Future studies should explore the extent and time course of molecular rejuvenation by iPSC reprogramming, to determine whether there is dependency between different age-associated features.

Recent studies using mouse models of doxycycline-inducible reprogramming factor (OSKM) expression have demonstrated that somatic cells can be reprogrammed to pluripotency in vivo[101-104], suggesting that the rejuvenating effects of cellular reprogramming might be recapitulated in an organism. A major limitation of initial studies was that persistent expression of OSKM led to teratoma formation[101-103]. Thus, an important step was to determine whether the rejuvenating aspect of reprogramming could be uncoupled from its dedifferentiating, teratoma-inducing properties[96]. Interestingly, this uncoupling was recently shown to be possible[105]. Short-term OSKM induction (‘partial reprogramming’) in fibroblasts from progeroid mice (*Lmna*^{G608G}) erased features of ageing, including DNA damage, dysregulation of histone marks, expression of senescence-associated genes and nuclear envelope abnormalities[105]. When applied in vivo, cyclic partial reprogramming (2-day induction with 5-day

withdrawal) starting at 8 weeks of age extended both healthspan and lifespan (median ~30%, maximum ~20%) of these mice, without teratoma or cancer development[105] (Fig. 1). In vivo partial reprogramming applied to naturally ageing mice at midlife also improved glucose tolerance and the regenerative capacity of muscle and the pancreas after injury[105] (Table 1 and Supplementary Table 1). These observations underscore the potential of cellular reprogramming to rejuvenate cells and tissues in vivo, although more work is needed in the context of naturally aged mice. Indeed, some of the positive OSKM effects in muscle at midlife could be age independent, as the regenerative potential of muscle has not declined yet at this stage of life[36,106]. Whether partial reprogramming can reverse tissue decline in the absence of injury or disease and/or extend lifespan in naturally aged mice also remains to be determined.

How does cellular reprogramming rejuvenate aged cells and tissues? At the molecular level, epigenetic remodelling is a key factor in iPSC reprogramming[107,108], and histone modifications have been proposed to mediate the rejuvenating effects of partial reprogramming[105] (Fig. 2). At the tissue level, partially reprogrammed mice have increased numbers of muscle stem cells after injury[105]. Hence, enhanced regenerative capacity and stem cell function could contribute to the lifespan extension observed in the context of premature ageing[105]. Reprogramming could also act by eliminating dysfunctional cells in tissues or by diluting them through proliferation of healthy cells. The extent to which rejuvenating effects persist after in vivo reprogramming remains an important direction for future studies. Although some reprogramming-induced epigenetic and transcriptomic remodelling persists following doxycycline withdrawal[101], the increase of histone 3 lysine 9 trimethylation (H3K9me3) levels reverts within 8 days of withdrawal in vitro[105]. Thus, whether transient reprogramming leads to transient or persistent rejuvenation remains to be determined.

Common or distinct mechanisms of rejuvenation

One key question is whether the four rejuvenation strategies described above share modes of action or whether they use distinct mechanisms (Fig. 2). Common pathways could be harnessed to induce rejuvenation more directly, whereas differing ones could be targeted in combination to enhance it.

Inflammation.

Inflammation could be directly or indirectly affected by most rejuvenation strategies. Heterochronic parabiosis reduces inflammatory factors and pathways, such as eotaxin and interferon signalling[21,31]. FMD and dietary restriction (DR)-mimicking drugs have anti-inflammatory effects by suppressing the onset of senescence and the secretion of pro-inflammatory cytokines[109-111]. Senolytics could exert their beneficial effects by reducing inflammation, as senescent cells contribute to inflammation through SASP[56,87]. Finally, although age-associated activation of nuclear factor-κB signalling impairs cellular reprogramming[112], activation of innate immunity and inflammatory factors, such as interleukin-6 (IL-6), promote reprogramming[102,104,113-115]. These observations

highlight inflammation as a critical target for rejuvenation strategies. Chronic inflammation ('inflammaging') has emerged as a key feature of ageing and age-associated diseases[1,2,116], and its genetic and pharmacological targeting has been shown to extend healthspan and lifespan across multiple species[117-121]. Interestingly, stimulation or blocking of hypothalamic nuclear factor-κB activity was shown to accelerate or decelerate ageing, respectively[122], suggesting a potential key role of the hypothalamus in modulating inflammation and ageing. Future studies should aim at investigating the interplay between rejuvenation strategies and inflammation, and exploring potential synergistic effects of rejuvenating compounds with anti-inflammatory drugs.

Nutrient-sensing pathways.

The insulin–IGF1, mTOR and AMPK pathways have been extensively studied in the context of longevity[1-3,45-47] and are key candidates for relaying rejuvenating effects. The anti-ageing diets discussed inhibit mTOR and/or elicit a drop in circulating insulin and IGF1 levels[5,8,9]. DR-mimicking drugs also inhibit insulin–IGF1 and mTOR signalling and activate AMPK[41,49,123]. However, evidence for the involvement of these pathways in heterochronic parabiosis, the elimination of senescent cells and cellular reprogramming is mostly circumstantial. The shared circulatory system and organs in parabiosis may affect glucose–insulin homeostasis and IGF1 signalling[124]. Moreover, the IGF1 and mTOR pathways promote senescent cell survival and regulate SASP[125-127], whereas AMPK pathway activation suppresses the development of senescence[128]. Finally, insulin–IGF1 signalling inhibits reprogramming[15,129,130], although the role of AMPK in cellular reprogramming is still debated[15]. An intriguing possibility is that nutrient-sensing pathways may be more important for delaying ageing than reversing it.

Epigenomic remodelling.

The epigenomic landscape of a cell reflects not only its identity but also its health and biological age[131-133]. Senescent cells exhibit a characteristic chromatin state[134,135], and their secreted factors (for example, IL-6) have been shown to induce epigenomic changes[136,137]. The rejuvenating effect of cellular reprogramming has been proposed to occur through epigenomic remodelling[105]. Moreover, dietary interventions and DR-mimicking drugs affect the epigenome[131,138,139], although whether these changes are necessary for rejuvenating effects is unclear. Finally, while chromatin changes have not yet been reported in the context of heterochronic parabiosis, chromatin changes could relay some effects[13]. Whether restoring a youthful epigenome holds the key to a prolonged rejuvenated state is a compelling question.

Autophagy.

Autophagy, which includes the process of delivering damaged proteins and organelles to lysosomes for degradation, is key for cellular homeostasis[140] and could play an important role in mediating rejuvenation. Most diet regimens and DR-mimicking drugs induce autophagy[5,140,141], and the blood factor GDF11 was shown to enhance this

process[20]. Senescent cell ablation could eliminate autophagy-deficient cells[142,143]. Finally, autophagy is also induced early in the reprogramming process[129]. Whether autophagy is necessary for the rejuvenation effects of cellular reprogramming remains unclear[15,129], but reactivation of the lysosome–autophagy pathway in aged stem cells improves their function[144-146]. These observations suggest a link between the lysosome–autophagy pathway and rejuvenation strategies, but the extent to which autophagy promotes rejuvenation remains to be explored.

Mitochondria.

Mitochondrial function could also be central to rejuvenation strategies. Cellular reprogramming increases mitochondrial fitness[98,147] and GDF11 can improve mitochondrial morphology and function[20]. Senescent cells have dysfunctional mitochondria with increased generation of reactive oxygen species, which in turn promote SASP and can induce senescence in neighbouring cells[148-150]. Mitochondria in senescent cells were recently suggested to have reduced ability to metabolize fatty acids, contributing to increased hepatic fat deposition with age and a decline in liver function[64]. Hence, the removal of senescent cells with poor mitochondrial function could be beneficial by reducing reactive oxygen species levels in the microenvironment and perhaps also by improving overall mitochondria function in ageing tissues and organs. However, rejuvenation strategies could also act by reducing mitochondrial function. Indeed, reduced mitochondrial activity extends lifespan in *Caenorhabditis elegans*, *Drosophila* and mice[151-155]. In addition, metformin, which inhibits mitochondrial function[141], can extend healthspan and/or lifespan in multiple organisms[41,141]. Future studies should explore how this organelle relays the rejuvenating effects of these different strategies.

These observations suggest that the four rejuvenation strategies could act through common molecular pathways. However, the degree to which these pathways are modulated and whether each strategy targets them directly or indirectly remain unclear. Investigating the regulation and sequential order of these pathways following each intervention will help to identify the mechanisms that are critical for restoring youthfulness and that could be targeted for greater effect. Different rejuvenation approaches could also act via diverse mechanisms, which could be combined to achieve synergistic effects. Broader conceptual questions also remain: is the rejuvenation process the direct opposite sequence of events that lead to ageing? Do rejuvenation strategies target the root cause of ageing or simply its consequences? Can these interventions affect overall lifespan?

Target cells for rejuvenation

Which cell types are primarily targeted by rejuvenation strategies and mediate their beneficial effects? Adult stem cells are an attractive candidate as they provide a renewable source of cells to repair damaged tissues (Fig. 2). Indeed, most rejuvenation approaches also improve stem cell functions[5,17,21,22,25,65,105,110,156], although whether these effects are direct or indirect remains unclear. The inherent plasticity of stem cells may make them more susceptible to the rejuvenating effects of cellular reprogramming, for example.

Stem cell state may also dictate susceptibility to ageing and rejuvenation. For example, quiescent stem cells exhibit increased age-related features compared to actively proliferating stem cells[146,157], raising the possibility that quiescent cells might benefit more from rejuvenation strategies. In fact, a proliferative state could itself reset ageing features (for example, DNA damage and protein aggregates) in stem cell populations[146,157]. In addition, these rejuvenation strategies could indirectly affect stem cells. For example, young blood was proposed to enhance neurogenesis in aged mice by improving endothelial cells and thereby the vasculature of the neural stem cell niche[22]. Moreover, although the senolyte ABT293 is thought to improve aged haematopoietic and muscle stem cells by eliminating senescent stem cells[65], it could also act by clearing senescent niche cells, such as endothelial cells and fibroblasts. In line with this notion, niche endothelial cells were shown to contribute to haematopoietic stem cell ageing, and transplantation of young endothelial cells could partially reverse these changes[158]. Thus, the primary target of rejuvenation approaches may be vascular and connective tissue cells. As these cells are present throughout the organism, targeting them may have broader organismal effects. Teasing apart the effects of rejuvenation strategies on different cell types and states will help efforts to improve tissue function and health and could identify strategies to simultaneously target both differentiated and stem cell populations for enhanced treatments.

Other attractive candidate cells for rejuvenation are senescent cells. Beyond their direct elimination by genetic means or senolytics, senescent cells may also be targeted by other rejuvenation strategies. The pro-ageing factor eotaxin[21] has been associated with senescence[159], potentially linking the beneficial effect of senescent cell ablation to changes in systemic factors. Moreover, FMD, DR and DR-mimicking drugs suppress senescence onset and pro-inflammatory cytokine levels[109-111,160]. Although speculative, it is also plausible that the proliferation bursts induced by FMD or partial reprogramming could dilute and/or trigger senescent cell clearance. Indeed, many of the age-associated features that are reverted by partial reprogramming are related to senescence[105]. Cellular reprogramming has been suggested to rejuvenate senescent cells[97]. However, the relationship between reprogramming and senescence is complex. Reprogramming factors can trigger cellular senescence[161,162]; conversely, senescence promotes cellular plasticity of neighbouring cells through SASP (for example, IL-6)[102,104,115,163]. In line with these observations, induction of reprogramming factors for 7 days results in more

teratomas in aged mice than in young mice[102-104], possibly due to the presence of senescent cells in aged tissues. Finally, senescent cell removal using senolytic drugs or an inducible genetic system decreases in vivo reprogramming efficiency[115]. It will be interesting to elucidate the interplay between senolytic and reprogramming strategies for rejuvenation.

Potential trade-offs of rejuvenation

Ageing disrupts the balance of key biological processes that maintain organismal homeostasis and function. Hence, reversing it is not as simple as turning off these processes, but rather involves the need to restore a balance. For example, although age-associated senescence and/or chronic inflammation could impair tissue function, they are also critical for normal tissue repair and remodelling[59,60]. Accordingly, counteracting senescence and/or inflammation could reduce the ability of the organism to perform these processes (Fig. 1). Indeed, elimination of senescent cells impedes tissue repair and promotes tissue-specific fibrosis[59,60,91]. Similarly, DR-related interventions impair the immune response to infections and reduce wound healing[164], although refeeding after DR or DR-mimicking drugs can restore or even potentiate these responses[164,165]. DR regimens, when started too early, can also interfere with growth and fecundity and lead to amenorrhea and osteoporosis[12]. Importantly, excessive perturbation of a specific feature may ultimately lead to tumorigenesis and cancer progression. As senescence is a critical barrier against tumorigenesis[56], preventing its induction could increase cancer risk. Similarly, sustained expression of reprogramming factors could lead to tumour formation[101]. Senescent cells also exploit anti-apoptotic pathways, such as Bcl-2, that are important for the survival of healthy cells (for example, lymphocytes and platelets)[166-168]. Consequently, compounds that are used to target senescent cells (for example, pan-Bcl inhibitors) are also associated with gastrointestinal symptoms and haematopoietic system toxicity[83,84]. Hence, the risk/benefit ratio of these rejuvenation strategies must be taken into account before considering them as a viable anti-ageing treatment.

Future perspectives

There is now compelling evidence that the ageing process is plastic and that it is possible to revive aged cells and tissues. Although the four strategies discussed here have received much attention in recent years, other approaches may also turn out to have rejuvenating effects. Genetic perturbations such as the expression of telomerase in middle-aged and old-aged mice improves healthspan (for example, insulin sensitivity and osteoporosis) and extends median lifespan[169]. Similarly, life-long increased dosage of $p16^{INK4}$ and p53 can have beneficial effects to counter ageing[170-172]. Hence, inducible telomerase, p16 or p53 expression later in life could be future rejuvenation strategies. Environmental interventions that have benefits on healthspan and lifespan could also be leveraged for rejuvenation. For example, exercise improves hippocampal neurogenesis and muscle function in aged rodents[173-175]. Lowering core body temperature extends lifespan in invertebrates and African killifish[3,176-178] and even in mice[179]. Finally, the transfer of young microbiome in middle-aged killifish was recently shown to extend both

healthspan and lifespan[180]. However, whether these potential strategies revert ageing hallmarks or delay the appearance of such characteristics remains to be tested. It will also be interesting to determine whether key organs or systems, such as the hypothalamus, orchestrate ageing in a centralized manner by integrating environmental inputs and secreting systemic factors[36,122,181]. These systems could then be targeted to achieve whole-organism rejuvenation.

The question also emerges of whether rejuvenation interventions, which were mainly tested in mice, may benefit human health and longevity (Fig. 1). Metabolic approaches have reached furthest in testing this possibility and have shown promise in benefiting humans. FMD in individuals ranging from 20 to 70 years of age was shown to improve physiological readouts that are altered with age, including body weight, blood pressure, cholesterol and IGF1 levels[5,182]. FMD and DR-mimicking drugs, such as metformin and rapamycin, can improve risk factors associated with age-related diseases, such as cancer, diabetes and cardiovascular disease[182-185]. Clinical trials are underway using metformin and rapamycin to target ageing[141] (ClinicalTrials.gov identifiers: NCT02432287 and NCT02874924) and rapamycin analogues are being tested in the elderly in the context of response to vaccination[165] and respiratory tract infection (ClinicalTrials.gov identifier: NCT03373903). Currently, there are no data showing beneficial effects of blood factors, senolytic drugs or reprogramming in humans. However, the levels of the pro-ageing blood factors eotaxin and β_2-microglobulin are increased in the plasma of elderly humans[21,23] and the rejuvenation factor TIMP2 is enriched in human umbilical plasma[26]. Moreover, most senolytic drugs identified can eliminate human senescent cells in vitro[61,65,66,68,85,186]. Similarly, cellular reprogramming can revert ageing features of human cells in vitro[95,97-100,105], raising the possibility that these approaches may also prove beneficial for human ageing. Indeed, some of these approaches are now being explored in the context of human age-associated diseases. For instance, young blood is being tested in Alzheimer's disease (ClinicalTrials.gov identifier: NCT02256306). Although the initial trial showed only a minor improvement[187], larger trials are underway to better assess efficacy. Several senolytics are currently used in the clinic as anticancer drugs[84,86] and are being tested on chronic kidney disease (ClinicalTrials.gov identifier: NCT02848131) and osteoarthritis (ClinicalTrials.gov identifier: NCT03513016). Initial findings are encouraging, but many challenges remain before these strategies can be used successfully in the clinic. The optimization of therapeutic dosage with minimal side effects will be key to translational efforts. It will also be critical to establish reasonable end points and robust biomarkers of healthy ageing to assess intervention efficacy.

These studies provide compelling evidence that the ageing process is malleable and that it is possible to revive aged cells, tissues and organs. They also raise the exciting possibility of translation to address human ageing and age-associated diseases. The coming years will undoubtedly see exciting developments in ongoing efforts to better understand, delay and potentially reverse ageing.

Telomere extension turns back aging clock in cultured human cells, study finds

Researchers delivered a modified RNA that encodes a telomere-extending protein to cultured human cells. Cell proliferation capacity was dramatically increased, yielding large numbers of cells for study.

A new procedure can quickly and efficiently increase the length of human telomeres, the protective caps on the ends of chromosomes that are linked to aging and disease, according to scientists at the Stanford University School of Medicine.

Treated cells behave as if they are much younger than untreated cells, multiplying with abandon in the laboratory dish rather than stagnating or dying.

The procedure, which involves the use of a modified type of RNA, will improve the ability of researchers to generate large numbers of cells for study or drug development, the scientists say. Skin cells with telomeres lengthened by the procedure were able to divide up to 40 more times than untreated cells. The research may point to new ways to treat diseases caused by shortened telomeres.

Telomeres are the protective caps on the ends of the strands of DNA called chromosomes, which house our genomes. In young humans, telomeres are about 8,000-10,000 nucleotides long. They shorten with each cell division, however, and when they reach a critical length the cell stops dividing or dies. This internal "clock" makes it difficult to keep most cells growing in a laboratory for more than a few cell doublings.

'Turning back the internal clock'

"Now we have found a way to lengthen human telomeres by as much as 1,000 nucleotides, turning back the internal clock in these cells by the equivalent of many years of human life," said Helen Blau, PhD, professor of microbiology and immunology at Stanford and director of the university's Baxter Laboratory for Stem Cell Biology. "This greatly increases the number of cells available for studies such as drug testing or disease modeling."

A paper describing the research was published today in the *FASEB Journal*. Blau, who also holds the Donald E. and Delia B. Baxter Professorship, is the senior author. Postdoctoral scholar John Ramunas, PhD, of Stanford shares lead authorship with Eduard Yakubov, PhD, of the Houston Methodist Research Institute.

The researchers used modified messenger RNA to extend the telomeres. RNA carries instructions from genes in the DNA to the cell's protein-making factories. The RNA used in this experiment contained the coding sequence for TERT, the active component of a naturally occurring enzyme called telomerase. Telomerase is expressed by stem cells, including those that give rise to sperm and egg cells, to ensure that the telomeres of these cells stay in tip-top shape for the next generation. Most other types of cells, however, express very low levels of telomerase.

Transient effect an advantage

The newly developed technique has an important advantage over other potential methods: It's temporary. The modified RNA is designed to reduce the cell's immune response to the treatment and allow the TERT-encoding message to stick around a bit longer than an unmodified message would. But it dissipates and is gone within about 48 hours. After that time, the newly lengthened telomeres begin to progressively shorten again with each cell division.

The transient effect is somewhat like tapping the gas pedal in one of a fleet of cars coasting slowly to a stop. The car with the extra surge of energy will go farther than its peers, but it will still come to an eventual halt when its forward momentum is spent. On a biological level, this means the treated cells don't go on to divide indefinitely, which would make them too dangerous to use as a potential therapy in humans because of the risk of cancer.

This new approach paves the way toward preventing or treating diseases of aging.

The researchers found that as few as three applications of the modified RNA over a period of a few days could significantly increase the length of the telomeres in cultured human muscle and skin cells. A 1,000-nucleotide addition represents a more than 10 percent increase in the length of the telomeres. These cells divided many more times in the culture dish than did untreated cells: about 28 more times for the skin cells, and about three more times for the muscle cells.

"We were surprised and pleased that modified TERT mRNA worked, because TERT is highly regulated and must bind to another component of telomerase," said Ramunas. "Previous attempts to deliver mRNA-encoding TERT caused an immune response against telomerase, which could be deleterious. In contrast, our technique is nonimmunogenic. Existing transient methods of extending telomeres act slowly, whereas our method acts over just a few days to reverse telomere shortening that occurs over more than a decade of normal aging. This suggests that a treatment using our method could be brief and infrequent."

Potential uses for therapy

“This new approach paves the way toward preventing or treating diseases of aging,” said Blau. “There are also highly debilitating genetic diseases associated with telomere shortening that could benefit from such a potential treatment.”

Blau and her colleagues became interested in telomeres when previous work in her lab showed that the muscle stem cells of boys with Duchenne muscular dystrophy had telomeres that were much shorter than those of boys without the disease. This finding not only has implications for understanding how the cells function — or don’t function — in making new muscle, but it also helps explain the limited ability to grow affected cells in the laboratory for study.

The researchers are now testing their new technique in other types of cells.

“This study is a first step toward the development of telomere extension to improve cell therapies and to possibly treat disorders of accelerated aging in humans,” said John Cooke, MD, PhD. Cooke, a co-author of the study, formerly was a professor of cardiovascular medicine at Stanford. He is now chair of cardiovascular sciences at the Houston Methodist Research Institute.

“We’re working to understand more about the differences among cell types, and how we can overcome those differences to allow this approach to be more universally useful,” said Blau, who also is a member of the Stanford Institute for Stem Cell Biology and Regenerative Medicine.

“One day it may be possible to target muscle stem cells in a patient with Duchenne muscular dystrophy, for example, to extend their telomeres. There are also implications for treating conditions of aging, such as diabetes and heart disease. This has really opened the doors to consider all types of potential uses of this therapy.”

Other Stanford co-authors of the paper are postdoctoral scholars Jennifer Brady, PhD, and Moritz Brandt, MD; senior research scientist Stéphane Corbel, PhD; research associate Colin Holbrook; and Juan Santiago, PhD, professor of mechanical engineering.

The work was supported by the National Institutes of Health (grants R01AR063963, U01HL100397 U01HL099997 and AG044815), Germany’s Federal Ministry of Education and Research, Stanford Bio-X and the Baxter Foundation.

Ramunas, Yakubov, Cooke and Blau are inventors on patents for the use of modified RNA for telomere extension.

Information about Stanford’s Department of Microbiology and Immunology, which also supported the work, is available at http://microimmuno.stanford.edu.

Mitochondria makeover: unlocking the path to healthy longevity

1. Introduction

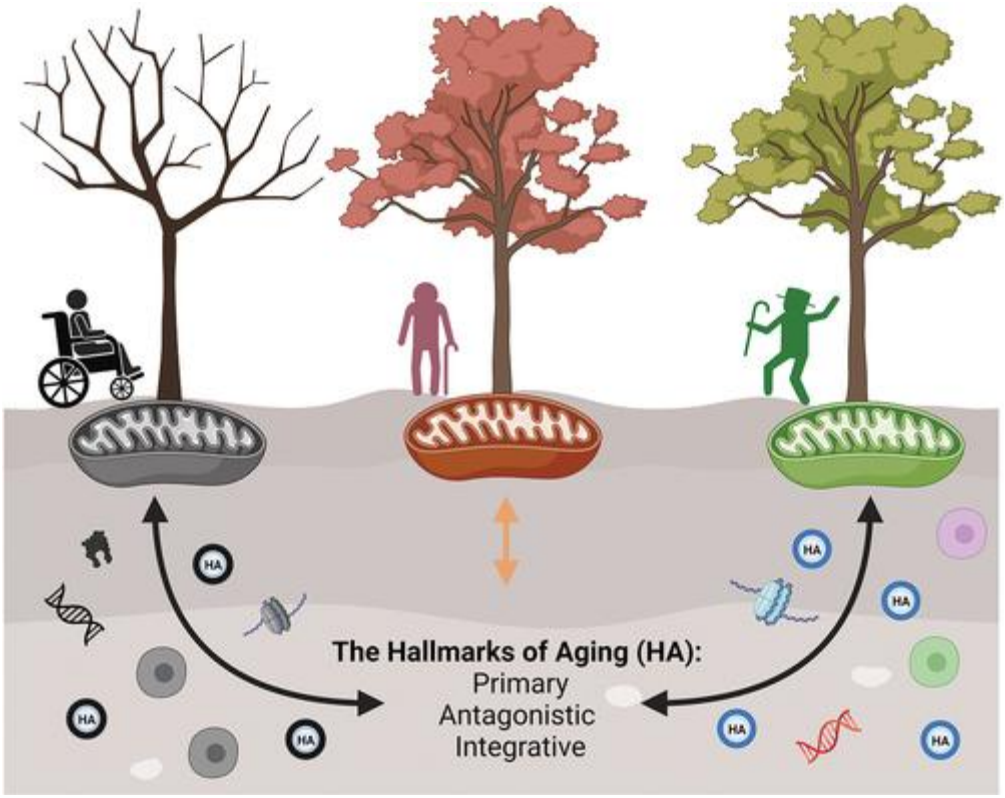
Thanks to biomedical research, significant advances have been made in preventing deaths caused by infectious agents and chronic diseases. Scientific progress has contributed to a remarkable increase in life expectancy, soaring from an estimated 30 years around 1800 to nearly 73 years by 2019 [Citation1]. This unprecedented global rise in life expectancy has resulted in a noticeable shift in the age distribution toward older demographics. However, even with these advancements, we still experience frailty as we age, accompanied by disabilities that reduce our quality of life. While the prospect of an extended lifespan is generally viewed positively, it is essential to recognize that living longer does not automatically guarantee good health. Therefore, developing interventions aimed at promoting healthy longevity has become imperative.

Achieving healthy longevity and mitigating the progressive loss of physiological integrity is a complex endeavor influenced by various factors that contribute to the development of vulnerability and diseases over time [Citation2]. In 2013, Lopez-Otin et al. proposed nine hallmarks of aging, sparking the publication of more than 300,000 articles with the aim of dissecting each hallmark to understand the molecular changes and develop interventions to mitigate aging and aging-associated diseases [Citation3]. Recently 12 hallmarks of aging were described, taking into account age-associated alterations in molecular, cellular, and systemic processes related to the deterioration of biological function over time. These hallmarks include primary (genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, and disabled macroautophagy), antagonistic (deregulated nutrient-sensing, cellular senescence, and mitochondrial dysfunction), and integrative (stem cell exhaustion, altered intercellular communication, chronic inflammation, and dysbiosis) [Citation3]. Notably among these hallmarks is mitochondria, which are interconnected with all of them, potentially offering a pathway to healthy longevity.

Today, our understanding of mitochondria extends beyond cellular boundaries, as they have been identified as viable entities outside of cells existing in circulation [Citation4–6]. Mitochondria transfer seems to play crucial roles in energy

management and facilitating inter-organ metabolic adaptation, particularly in response to nutrient stress and possibly caloric restriction [Citation7]. The phenomenon of natural (natural mitochondrial transfer, NMT) and artificial mitochondrial transfer/transplant (AMT/T) between cells opens up the possibility of modifying the metabolism, mitochondrial DNA (mtDNA), and phenotype of recipient cells, particularly those that could be affected by the aging process [Citation8,Citation9]. This suggests that mitochondria, with their multifaceted roles in intracellular molecular interactions, cellular function, and systemic effects by their transfer between cells, may occupy a central position in the study of the hallmarks of aging and the development of interventions (Figure 1). We focus on uncovering and proposing an untested link between the hallmarks of aging and mitochondrial transfer to promote healthy longevity.

Figure 1. Mitochondria exert an influence on the hallmarks of aging, while the hallmarks, in turn, reciprocally impact mitochondria, shaping the aging process. Trees symbolize the diverse ways we experience aging, and they are intricately linked to mitochondrial function. Mitochondrial function, in turn, is influenced by the hallmarks of aging, which can also be influenced by mitochondria. The hallmarks of aging are categorized into three groups: primary (genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, and disabled macroautophagy), antagonistic (deregulated nutrient-sensing), and integrative (cellular senescence, stem cell exhaustion, altered intercellular communication, chronic inflammation, and dysbiosis). The hallmarks of aging, much like soil, can be differently enriched, leading to changes in mitochondrial function. Created with BioRender.com.



2. Mitochondrial dysfunction: a central hub linking all other hallmarks of aging

Mitochondria serve as a central hub connecting all other hallmarks of aging and encompassing both their well-understood functions and those crucially remain to be described, such as mitochondrial transfer between cells [Citation10,Citation11]. In light of recent advances in interconnected pathways that may contribute to longevity and reduced age-associated diseases, mitochondria emerge as a key factor of aging decay. Furthermore, it is crucial to delve into how these factors could influence the release and uptake of our 'nomad' mitochondria as they migrate outside the cell and from one cell to another, enriching recipient cells with healthy mitochondria and potentially reducing effects on the aging hallmarks.

Single nucleotide polymorphisms (SNPs) in mtDNA have been associated with healthy longevity in centenarians, providing insights into their key role in the aging process and the importance of genomic stability [Citation12]. In contrast, genomic instability is related to the accumulation of somatic mutations in nuclear and mitochondrial DNA affecting essential genes and transcriptional pathways, leading to cellular dysfunction and compromising our health. Depletion in mtDNA content

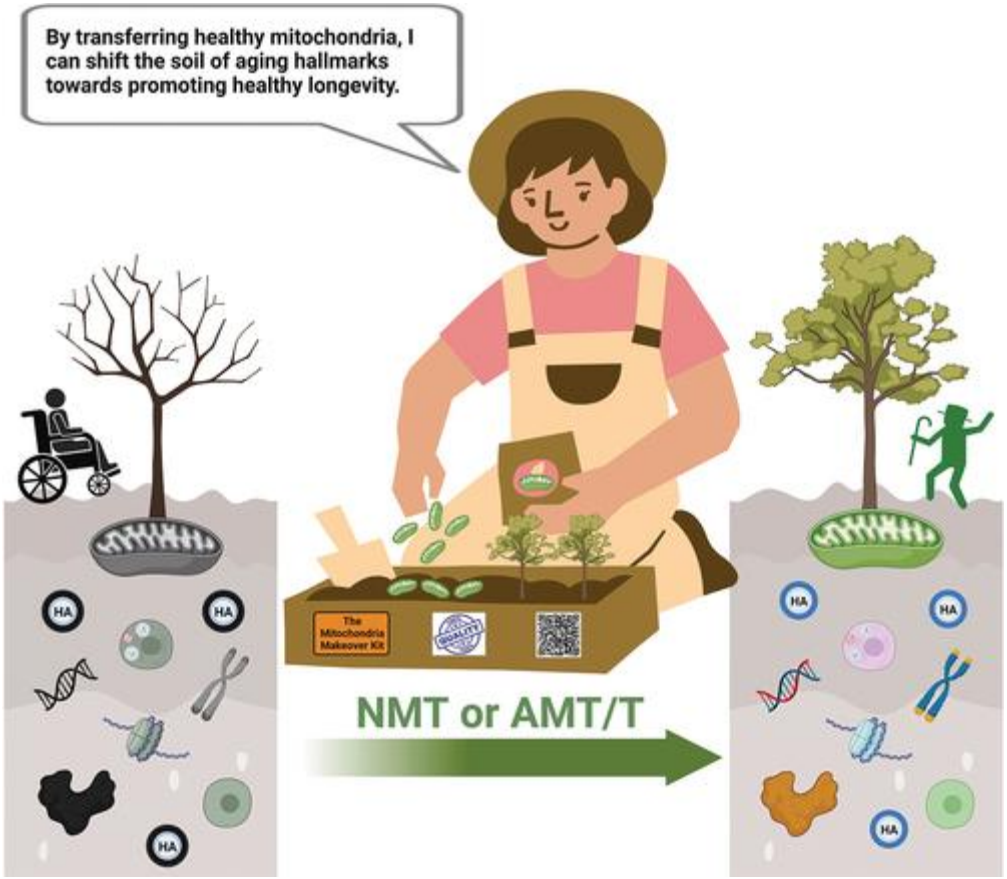
and mitochondrial number is reported during aging [Citation13]. Low mtDNA copy number correlates with frailty and all-cause mortality. Recent research indicates an average loss of four mtDNA copies per decade in humans, linked to age-related physiological changes [Citation14–16]. Additionally, numerous studies affirm the connection between reduced mtDNA content and aging and aging-related diseases [Citation13]. Since the pioneering study conducted by Clark and Shay in 1982, AMT/T has enabled the selective enrichment of a cell's mitochondrial content with a specific type conferring a selective advantage to cells that are less fit or have become unhealthy as a result of the aging process [Citation6,Citation17].

Among the primary hallmarks of aging, the connection between telomere damage and mitochondrial dysfunction is pivotal to understanding cellular decay over time. The relationship between telomere attrition and mitochondrial metabolic malfunction, particularly through the p53-PGC-1α-NRF-1 axis, remains a subject requiring further research [Citation18]. Interestingly, during the reprogramming of induced pluripotent stem cells (iPSCs), which is associated with potential rejuvenation, telomerase is upregulated, resulting in the lengthening of telomeres to embryonic stem cell (ESC)-like lengths. However, mitochondria that have accumulated mutations during the previous somatic life of the iPSCs exhibit diverse responses, leading to the generation of various mtDNA variants and mitochondrial heterogeneity [Citation19]. Some iPSCs appear to be better suited than others, with specific mtDNA variants improving reprogramming efficiency [Citation20]. The question arises: Is it possible to enhance the reprogramming process using specific AMT/T of mitochondria with mtDNA variants to effectively reset the cellular clock and rejuvenate cells? This intriguing avenue of research holds promise for unlocking new insights into cellular rejuvenation and healthy aging.

The other primary, antagonistic and the integrative hallmarks,—altered intercellular communication, stem cell exhaustion, and chronic inflammation – could also be mediated by NMT and AMT/T of healthy mitochondria. Mesenchymal stem cells (MSCs), along with the hematopoietic niche, are key components of tissue stability and function. Currently, it is unknown how MSCs are affected with time and whether the aging process could change their capacity to transfer mitochondria naturally to other cells. NMT and AMT/T could have strong implications for how cells communicate with each other to maintain their function over time and preserve the health of the immune system (Figure 2). Furthermore, observations indicate that the transfer of mitochondria to immune cells, whether through natural processes or via AMT/T from MSCs, can induce an immunoregulatory profile and potentially reduce chronic inflammation. Indeed, Singh et al. (2018) have shown

that restoring mitochondrial function can mitigate the aging process in mice [Citation13].

Figure 2. Mitochondria, playing critical roles in molecular, cellular, and systemic aspects of aging, have the potential to profoundly impact the hallmarks of aging (HA) through both natural mitochondrial transfer (NMT) and artificial mitochondrial transfer/transplant (AMT/T). Mitochondria play a pivotal role in governing the molecular, cellular, and systemic processes associated with aging, both in accelerating and potentially reversing the aging process for healthy longevity. In addition to their vital intracellular functions, both NMT and AMT/T hold promise as tools for gaining deeper insights into the hallmarks of aging and for the development of therapeutic interventions. Created with BioRender.com.



It's worth noting that, except for a few exceptions, the spectrum, precise nature and extent of mitochondrial dysfunction concerning the hallmarks of aging remain largely unknown across various cell types and tissues. The factors primarily responsible for the decline in the common core components of the mitochondrial metabolic machinery, leading to dysfunction and subsequent aging in different

tissues, organs, and the entire organism, remain unclear. Intriguingly, not all tissues age in the same manner, adding further complexity to this phenomenon. It could be possible to stimulate NMT between cells in a particular tissue or harness AMT/T with tissue-specific administration of mitochondria. This approach should lead to the utilization of specific mitochondrial transfers in tissues that are particularly affected, offering innovative strategies to rejuvenate and mitigate tissue-specific aging (Figure 2).

Targeting mitochondria and facilitating NMT or AMT/T as a primary approach to extend lifespan and delay aging-associated disease onset necessitates more research. This research should span from basic science to translational and clinical validation. The identification and understanding of core similarities and tissue-specific differences in the nature and scope of mitochondrial dysfunction, along with its connections to the hallmarks of aging, hold the potential to significantly advance our knowledge in the field of healthy longevity (Figure 2).

3. Expert opinion

In the pursuit of achieving healthy longevity, we have discovered that intricate interactions among aging hallmarks converge upon a central player – mitochondria. This dynamic organelle, long recognized for its pivotal role in energy production, management, and metabolism, has now emerged as a linchpin connecting the dots among distinct aging hallmarks. From genomic instability to telomere attrition, from cellular senescence to chronic inflammation, mitochondria stand at the nexus of these age-related processes. Recent breakthroughs in our understanding of NMT and AMT/T have illuminated a path toward restoring mitochondrial function, regenerating cellular vitality, and rejuvenating aging cells, tissues, and organs. These ‘nomad’ mitochondria, migrating between cells, enriching recipient cells with essential functions, and potentially mitigating the effects of other aging hallmarks, present new strategies for extending healthy lifespans (Figure 2). While it is known that the restoration of mitochondrial function can reverse various aging phenotypes [Citation13], it is currently unknown whether cellular rejuvenation through mitochondrial transfer/transplantation can fully reverse the effects of aging. As research advances and synthetic biology opens up new possibilities, we may harness the power of custom-designed mitochondria to re-energize aging tissues and bolster our health over time. In the ever-evolving landscape of aging research, mitochondria must take center stage, offering the prospect of a healthier, more resilient journey through the passage of time.

Declaration of interest

A Caicedo is the leader of Dragon BioMed. KK Singh is the scientific founder of Yuva Biosciences and serves as Chief Scientific Advisor. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

Author contributions

A Caicedo and KK Singh wrote the manuscript, cured, analyze and conveyed the information in the manuscript. A Caicedo and KK Singh proposed natural mitochondrial transfer (NMT) and artificial mitochondrial transfer/transplant (AMT/T) as key mitigators of aging hallmarks. A Caicedo made the figures with comments from KK Singh. A Caicedo and KK Singh conceived this work.

Acknowledgments

We would like to express our gratitude to the School of Medicine at the Universidad San Francisco de Quito (USFQ), the 'Instituto de Investigaciones en Biomedicina, USFQ,' and the Mito-Act Research Consortium in Quito, Ecuador, for their unwavering support of our work and initiatives. Andres Caicedo extends his gratitude to Luisa Páliz for her invaluable support in his personal development and career. AC and KKS thank their laboratory members for their participation in research targeting mitochondria to mitigate aging and aging-associated diseases. The authorship team thanks Reema Azar for her help in ensuring the appropriate use of the English language in the manuscript.

Photobiomodulation: The Clinical Applications of Low-Level Light Therapy

Graeme Ewan Glass[1]

- PMID: 33471046
- DOI: 10.1093/asj/sjab025

Erratum in

- Correction to: Photobiomodulation: The Clinical Applications of Low-Level Light Therapy.
  Glass GE.Aesthet Surg J. 2022 Apr 12;42(5):566. doi: 10.1093/asj/sjab396.PMID: 35043142 No abstract available.

Abstract

Background: Low-level light therapy (LLLT) is a recent addition to the pantheon of light-based therapeutic interventions. The absorption of red/near-infrared light energy, a process termed "photobiomodulation," enhances mitochondrial ATP production, cell signaling, and growth factor synthesis, and attenuates oxidative stress. Photobiomodulation is now highly commercialized with devices marketed directly to the consumer. In the gray area between the commercial and therapeutic sectors, harnessing the clinical potential in reproducible and scientifically measurable ways remains challenging.

Objectives: The aim of this article was to summarize the clinical evidence for photobiomodulation and discuss the regulatory framework for this therapy.

Methods: A review of the clinical literature pertaining to the use of LLLT for skin rejuvenation (facial rhytids and dyschromias), acne vulgaris, wound healing, body contouring, and androgenic alopecia was performed.

Results: A reasonable body of clinical trial evidence exists to support the role of low-energy red/near-infrared light as a safe and effective method of skin rejuvenation, treatment of acne vulgaris and alopecia, and, especially, body contouring. Methodologic flaws, small patient cohorts, and industry funding mean there is ample scope to improve

the quality of evidence. It remains unclear if light-emitting diode sources induce physiologic effects of compararable nature and magnitude to those of the laser-based systems used in most of the higher-quality studies.

Conclusions: LLLT is here to stay. However, its ubiquity and commercial success have outpaced empirical approaches on which solid clinical evidence is established. Thus, the challenge is to prove its therapeutic utility in retrospect. Well-designed, adequately powered, independent clinical trials will help us answer some of the unresolved questions and enable the potential of this therapy to be realized.

Comment in

- Commentary on: Photobiomodulation: The Clinical Applications of Low Level Light Therapy.
  Sun Y, Lan CE.Aesthet Surg J. 2021 May 18;41(6):739-740. doi: 10.1093/asj/sjaa394.PMID: 33647952 No abstract available.

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November 13th, 2015

Investigating Mitochondrial Rejuvenation During Cellular Reprogramming and Embryonic Development

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The changes involved in producing induced pluripotent stem cells from ordinary somatic cells, such as those from a skin sample, are accompanied by mitochondrial rejuvenation, a clearance of mitochondrial damage associated with aging. This also occurs in the earliest stages of embryonic development, turning old parental cells into young child cells. It is not beyond the bounds of the possible to suggest that perhaps just this mitochondrial part of the transformation could be split off and used as the basis for a therapy - though other approaches to mitochondrial repair are far closer to realization. Also, it may well be that mitochondria are so vital to cellular function that it is impossible to safely induce such radical changes in adult tissues given the way in which cells are presently structured. As usual, the only way to find out is to dig deeper into what is going on under the hood, as researchers are doing here. The original research release is in PDF format only, unfortunately, but it provides a better explanation than any of the other available resources:

A new study suggests that old mitochondria - the oxygen-consuming metabolic engines in cells - are roadblocks to cellular rejuvenation. By tuning up a gene called Tcl1, which is highly abundant in eggs, researchers were able to suppress old mitochondria to enhance a process known as somatic reprogramming, which turn adult cells into embryonic-like stem cells. Researchers found that Tcl1 does its job by suppressing mitochondrial polynucleotide phosphorylase (PnPase), thereby inhibiting mitochondrial growth and metabolism.

Stem cell researchers had known that egg (or oocyte) cytoplasm contains some special unknown factors that can reprogramme adult cells into embryonic-like stem cells, either during egg-sperm fertilisation or during artificial cloning procedures. While researchers had invented a technology

called induced pluripotent stem cell (iPSC) reprogramming to replace the ethically controversial oocyte-based reprogramming technique, oocyte-based reprogramming was still deemed superior in complete cellular reprogramming efficiency. To address this shortfall, researchers combined oocyte factors with the iPSC reprogramming system. Their bioinformatics-driven screening efforts1 led to two genes: Tcl1 and its cousin Tcl1b1. After a deeper investigation, the team found that the Tcl1 genes were acting via the mitochondrial enzyme, PnPase. "We were quite surprised, because nobody would have thought that the key to the oocyte's reprogramming powers would be a mitochondrial enzyme. The stem cell field's conventional wisdom suggests that it should have been some other signalling genes instead."

Tcl1 is a cytoplasmic protein that binds to the mitochondrial enzyme PnPase. By locking PnPase in the cytoplasm, Tcl1 prevents PnPase from entering mitochondria, thereby suppressing its ability to promote mitochondrial growth and metabolism. Thus, an increase in Tcl1 suppresses old mitochondria's growth and metabolism in adult cells, to enhance the somatic reprogramming of adult cells into embryonic-like stem cells. These new insights could boost efficacy of the alternative, non-oocyte based iPSC techniques for stem cell banking, organ and tissue regeneration, as well as further our understanding of how cellular metabolism rejuvenates after egg-sperm fertilisation.

Effect of Pulsing in Low-Level Light Therapy

Javad T Hashmi [1], Ying-Ying Huang [1,2,3], Sulbha K Sharma [1], Divya Balachandran Kurup [1], Luis De Taboada [4], James D Carroll [5], Michael R Hamblin [1,2,5,6,*]
PMCID: PMC2933784 NIHMSID: NIHMS232375 PMID: 20662021
The publisher's version of this article is available at Lasers Surg Med

Abstract

Background and Objective

Low level light (or laser) therapy (LLLT) is a rapidly growing modality used in physical therapy, chiropractic, sports medicine and increasingly in mainstream medicine. LLLT is used to increase wound healing and tissue regeneration, to relieve pain and inflammation, to prevent tissue death, to mitigate degeneration in many neurological indications. While some agreement has emerged on the best wavelengths of light and a range of acceptable dosages to be used (irradiance and fluence), there is no agreement on whether continuous wave or pulsed light is best and on what factors govern the pulse parameters to be chosen.

Study Design/Materials and Methods

The published peer-reviewed literature was reviewed between 1970 and 2010.

Results

The basic molecular and cellular mechanisms of LLLT are discussed. The type of pulsed light sources available and the parameters that govern their pulse structure are outlined. Studies that have compared continuous wave and pulsed light in both animals and patients are reviewed. Frequencies used in other pulsed modalities used in physical therapy and biomedicine are compared to those used in LLLT.

Conclusion

There is some evidence that pulsed light does have effects that are different from those of continuous wave light. However further work is needed to define these effects for different disease conditions and pulse structures.
Keywords: low level light therapy, photobiomodulation, frequency, pulse duration, duty cycle, clinical trials

INTRODUCTION

Since the introduction of low-level laser (light) therapy in 1967, over two hundred randomized, double-blinded, and placebo-controlled phase III clinical trials have been published from over a dozen countries. Whereas there is some degree of consensus as to the best wavelengths of light and acceptable dosages to be used, there is no

agreement on whether continuous wave (CW) or pulsed wave (PW) light is more suitable for the various applications of LLLT. This review will raise (but not necessarily answer) several questions. How does pulsed light differ from CW on the cellular and molecular level, and how is the outcome of LLLT affected? If pulsing is more efficacious, then at what pulse parameters is the optimal outcome achieved? In particular, what is the ideal pulse repetition rate or frequency to use?

PULSE PARAMETERS AND LIGHT SOURCES

There are five parameters that could be specified for pulsed light sources. The pulse width or duration or ON time (PD) and the pulse Interval or OFF time (PI) are measured in seconds. Pulse repetition rate or frequency (F) is measured in Hz. The duty cycle (DC) is a unitless fractional number or %. The peak power and average power are measured in Watts.

Pulse duration, pulse repetition rate, and duty cycle are related by the simple equation:

$$DC=F\times PD$$

Peak power is a measure of light intensity during the pulse duration, and related to the average power (measured in Watts) by:

$$Averagepower=Peakpower\times F\times PD$$

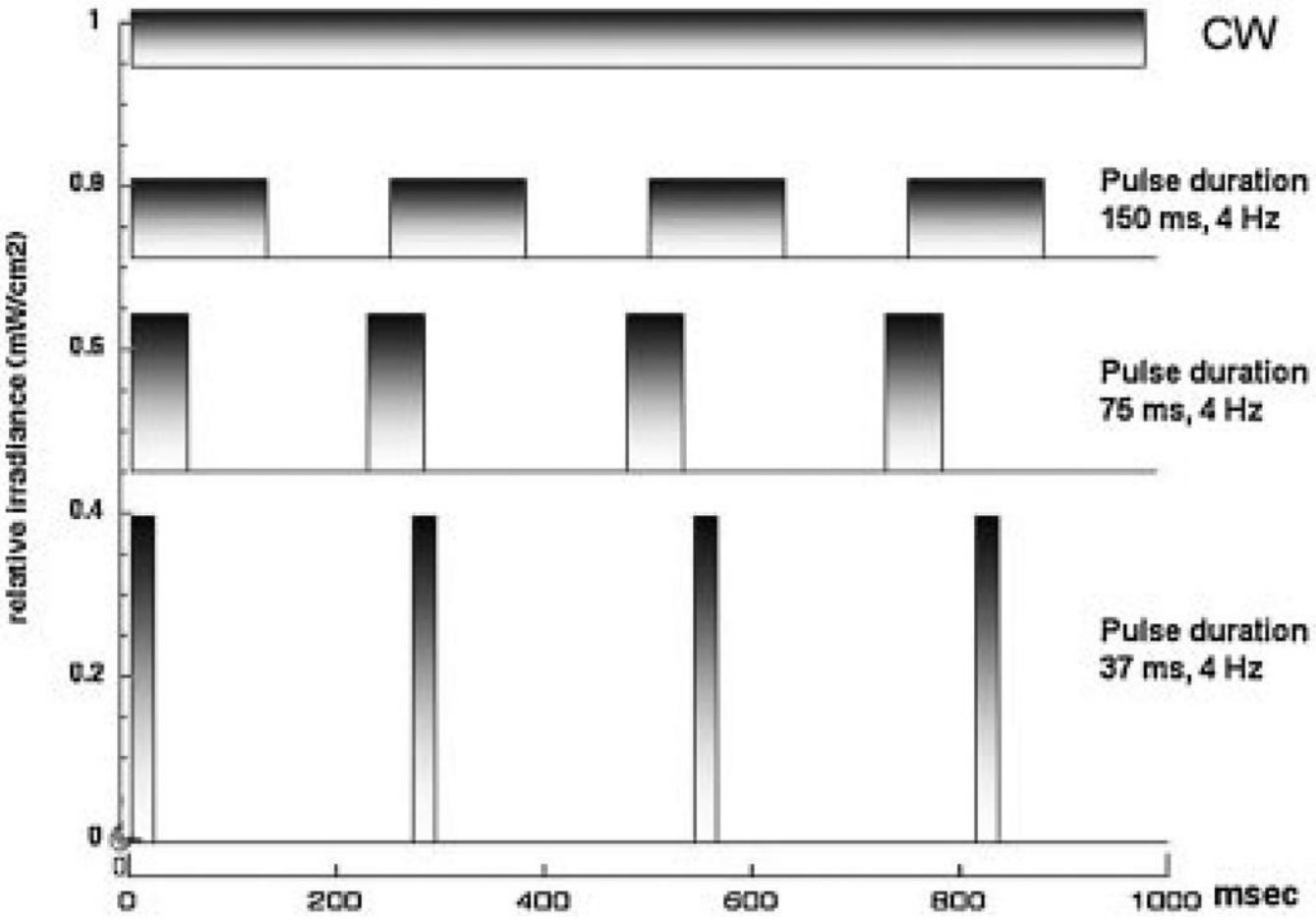
Alternatively,

$$Peakpower=AveragepowerDC$$

In all cases, it is necessary to specify any two out of three of: PD, F, and DC, and either the peak or average power for the pulse parameters to be fully defined.

Figure 1 graphically shows the relationship between peak power and pulse duration.

Fig. 1.



Conceptual diagram comparing the structure of CW with pulsed light of various pulse durations.

TYPES OF PULSED LIGHT SOURCES

Five major types of pulsed lasers (or other light sources) are commonly utilized: (1) Q-switched, (2) Gain-switched, (3) Mode-locked, (4) Superpulsed, and (5) Chopped or gated. Each utilizes a different mechanism to generate light in a pulsed as opposed to continuous manner, and vary in terms of pulse repetition rates, energies, and durations. However the first three classes of “truly” pulsed lasers mentioned above are in general not used for LLLT; instead superpulsed or gated lasers are mainly used. The concept of super-pulsing was originally developed for the carbon dioxide laser used in high power tissue ablative procedures. The idea was that by generating relatively short pulses (μsecond) the laser media could be excited to higher levels than those normally allowed in CW mode where heat dissipation constraints limit the maximum amounts of energy that can be used to excite the lasing media. With the original carbon dioxide superpulsed lasers, the short pulses would confine the thermal energy in the tissue (by making the pulse duration less than the thermal diffusion time) reducing collateral thermal damage to normal tissue.

Another type of laser that particularly benefited from super-pulsing is the gallium-arsenide (GaAs) diode laser. This laser has a wavelength in the region of 904-nm and pulse duration usually in the range of 100–200 nanoseconds. Another semiconductor laser amenable to superpulsing is the indium-gallium-arsenide (In-Ga-As) diode laser. It emits light at a similar wavelength (904–905-nm) as the GaAs diode laser, producing very brief pulses (200 nanoseconds) of high frequencies (in the range of kilohertz). These pulses are of very high peak powers (1–50 W) and an average power of 60 mW. Theoretically, the super-pulsed GaAs and In-Ga-As lasers allow for deep penetration without the unwelcome effects of CW (such as thermal damage), as well as allowing for shorter treatment times.

The other major class of pulsed light sources used in LLLT are simply CW lasers (usually diode lasers) that have a pulsed power supply generated by a laser driver containing a pulse generator. This technology is described as “chopped” or “gated.” It is also equally feasible to use pulse generator technology to pulse LEDs or LED arrays [1].

WHY COULD PULSING BE IMPORTANT IN LLLT?

Pulsed light offers numerous potential benefits. Because there are “quench periods” (pulse OFF times) following the pulse ON times, pulsed lasers can generate less tissue heating. In instances where it is desirable to deliver light to deeper tissues increased powers are needed to provide adequate energy at the target tissue. This increased power can cause tissue heating at the surface layers and in this instance pulsed light could be very useful. Whereas CW causes an increase in temperature of the intervening and target tissues or organ, pulsed light has been shown to cause no measurable change in the temperature of the irradiated area for the same delivered energy density. Anders et al. administered pulsed light to pig craniums, and found no significant change in temperature of the scalp or skull tissue (J.J. Anders, personal communication). Ilic et al. [2] found that pulsed light (peak power densities of 750 mW/cm^2) administered for 120 seconds produced no neurological or tissue damage, whereas an equal power density delivered by CW (for the same number of seconds) caused marked neurological deficits.

Aside from safety advantages, pulsed light might simply be more effective than CW. The “quench period” (pulse OFF times) reduces tissue heating, thereby allowing the use of potentially much higher peak power densities than those that could be safely used in CW. For example, when CW power densities at the skin of ≥2 W/cm^2 are used, doubling the CW power density would only marginally increase the treatment depth while potentially significantly increasing the risk of thermal damage; in contrast, peak powers of ≥5 W/cm^2 pulsed using appropriate ON and OFF times might produce little, or no tissue heating. The higher peak powers that can be safely used by pulsing light can overcome tissue heating problems and improve the ability of the laser to penetrate deep tissues achieving greater treatment depths.

There may be other biological reasons for the improved efficacy of pulsed light (PW) over CW. The majority of the pulsed light sources used for LLLT have frequencies in the 2.5–10,000 Hz range and pulse durations are commonly in the range of a few millisecond. This observation suggests that if there is a biological explanation of the improved effects of pulsed light it is either due to some fundamental frequency that exists in biological systems in the range of tens to hundreds of Hz, or alternatively due to some biological process that has a time scale of a few milliseconds. Two possibilities for what these biological processes could actually be occur to us. Firstly, it is known that mammalian brains have waves that have specific frequencies [3]. Electroencephalography studies have identified four distinct classes of brain waves [4,5]. Alpha waves (8–13 Hz) occur in adults who have their eyes closed or who are relaxed [6]. Beta waves (14–40 Hz) mainly occur in adults who are awake, alert or focused [7]. Delta waves (1–3 Hz) occur mainly in infants, adults in deep sleep, or adults with brain tumors [8]. Theta waves (4–7 Hz) occur mainly in children ages 2–5 years old and in adults in the twilight state between sleeping and waking or in meditation [9]. The possibility of resonance occurring between the frequency of the light pulses and the frequency of the brain waves may explain some of the results with transcranial LLLT using pulsed light.

Secondly, there are several lines of evidence that ion channels are involved in the subcellular effects of LLLT. Some channels permit the passage of ions based solely on their charge of positive (cationic) or negative (anionic) while others are selective for specific species of ion, such as sodium or potassium. These ions move through the channel pore single file nearly as quickly as the ions move through free fluid. In some ion channels, passage through the pore is governed by a “gate,” which may be opened or closed by chemical or electrical signals, temperature, or mechanical force, depending on the variety of channel. Ion channels are especially prominent components of the nervous system. Voltage-activated ion channels underlie the nerve impulse and while transmitter-activated or ligand-gated channels mediate conduction across the synapses.

There is a lot of literature on the kinetics of various classes of ion channels but in broad summary it can be claimed that the time scale or kinetics for opening and closing of ion channels is of the order of a few milliseconds. For instance Gilboa et al. [10] used pulses having a width 10 milliseconds and a period of 40 milliseconds (25 Hz). Other reports on diverse types of ion channels have given kinetics with timescales of 160 milliseconds [11], 3 milliseconds [12] and one paper giving three values of 0.1, 4 and 100 milliseconds [13]. Potassium and calcium ion channels in the mitochondria and the sarcolemma may be involved in the cellular response to LLLT [14–16].

Thirdly there is the possibility that one mechanism of action of LLLT on a cellular level is the photodissociation of nitric oxide from a protein binding site (heme or copper center) such as those found in cyctochrome *c* oxidase [17]. If this process occurs it is likely that the NO would rebind to the same site even in the presence of continuous light. Therefore if the light was pulsed multiple photodissociation events could occur, while in CW mode the number of dissociations may be much smaller.

PENETRATION DEPTH

The most important parameter that governs the depth of penetration of laser light into tissue is wavelength. Both the absorption and scattering coefficients of living tissues are higher at lower wavelength so near-infrared light penetrates more deeply that red and so on. It is often claimed that pulsed lasers penetrate more deeply into tissue than CW lasers with the same average power. Why exactly should this be so? Let us suppose that at a certain wavelength (for instance 810-nm) the depth of tissue at which the intensity of a laser is reduced to 10% of its value at the surface of the skin is 1-cm. Therefore if we are using a laser with a power density (irradiance) of 100 mW/cm^2 at the skin, the power density remaining at 1 cm below the skin is 10 mW/cm^2 and at 2-cm deep is 1 mW/cm^2. Now let us suppose that a certain threshold power density (minimum number of photons per unit area per unit time) at the target tissue is necessary to have a biological effect and that this value is 10 mW/cm^2. The effective penetration depth at CW may be said to be 1-cm. Now let us suppose that the laser is instead pulsed with a 10-milliseconds pulse duration at a frequency of 1 Hz (DC = 1 Hz×0.010 seconds = 0.010) and the same average power. The peak power and peak power densities are now 100 times higher (peak power = average power/DC = average power×100). With a peak power density of 10 W/cm^2 at the skin, the tissue depth—at which this peak power density is attenuated to the threshold level of 10 mW/cm^2—is now 3-cm rather than 1-cm in CW mode. But what we have to consider is that the laser is only on for 1% of the time so the total fluence delivered to the 3-cm depth in pulsed mode is 100 times less than that delivered to 1-cm depth in CW mode. However it would be possible to increase the illumination time by a factor of 100 to reach the supposed threshold of fluence as well as the threshold of power at the 3-cm depth. In reality the increase in effective penetration depth obtained with pulsed lasers is more modest than simple calculations might suggest. Many applications of LLLT do not require deep penetration such as tendinopathies and joint pain.

Similarly, deep penetration is often not required to alleviate joint pain. The target tissue in such cases is the synovia; with the exception of back, neck, and hip, most joints have readily accessible synovia. Bjordal et al. [19] conducted a review of literature and concluded that “superpulsed” lasers (904 nm) were not significantly more effective than CW lasers (810–830 nm); both types of laser achieved similar results, but half the energy was needed to be used for superpulsed lasers. On the other hand, deeper penetrance is needed to reach back, neck, and hip joints. If power densities greater than a few mW/cm^2 are to be safely delivered to target tissues >5 cm below the skin, it appears likely that this can only be done by using pulsed lasers. It is postulated that successful LLLT treatments in such joints bring benefit not by reaching the deep target tissue but by inhibiting superficial nociceptors. In other words, they bring relief primarily by attenuating pain perception, as opposed to decreasing inflammation. Does deeper penetration via pulsed lasers offer any significant benefit over CW? It is quite possible that a relatively higher fluence is necessary to attenuate pain, whereas a lower fluence decreases inflammation. If this is indeed the case, for musculo-skeletal applications achieving higher doses at the level of the target tissue may not be ideal. Further studies

Refs.	Subject	Condition	λ (nm)	*f* (Hz)	Other reported parameters	Results?
Kymplova et al. [24]	Humans	Wound healing	670	10, 25, and 50	Power: 20 mW; energy density: 2 J/cm^2	PW > CW
Brondon et al. [36]	In vitro (human HEP-2 cells)	Increasing the penetration depth of light through melanin filters	670	6, 18, 36, 100, and 600	Power: 10 mW; energy density: 5 J/cm^2	PW > CW
Lapchak et al. [26]	Rabbits	Ischemic stroke	808	100 and 1,000	Power density: 7.5 mW/cm^2; ON time: 0.3 milliseconds (1,000 Hz), 2 milliseconds (100 Hz); average energy delivered to the brain: 0.9–1.2 J; duty cycle: 30% and 20%	PW > CW
Lapchak and De Taboada [27]	Rabbits	Ischemic stroke	808	100	Cortical irradiance: 7.5 mW/cm^2 (CW), 37.5 mW/cm^2 (PW); cortical fluence: 0.9 J/cm^2 (CW), 4.5 J/cm^2 (PW)	PW > CW
Gigo-Benato et al. [23]	Rats	Nerve regeneration	808 (CW), 905 (PW), 808 + 905 (CW + PW)	10,000	Power: 416 mW (CW), 28 W (PW); energy density: 29 J/cm^2 (CW), 40 J/cm^2 (PW); pulse duration: 454 seconds (CW), 200 nanoseconds (PW)	Combined (CW + PW) > CW > PW
Braverman et al. [61]	Rabbits	Wound healing	632.8 (CW), 904 (PW)	4,672	Power: 10 mW (CW), 50 mW (PW); energy density: 1.65 J/cm^2 (CW), 8.25 J/cm^2 (PW); pulse duration: 200 nanoseconds	CW = PW
Al-Watban and Zhang [28]	Rats	Wound healing	635	100, 200, 300, 400, and 500	Power density: 0.89 mW/cm^2; energy density: 1.0 J/cm^2	CW > PW
Ueda and Shimizu [37]	In vitro (osteoblast-	Bone stimulation	830	1, 2, and 8	N/A	PW > CW

Refs.	Subject	Condition	λ (nm)	*f* (Hz)	Other reported parameters	Results?
	like cells from fetal rat calvariae)					
Sushko et al. [25]	Mice	Pain	610–670, 850–910	10, 600, and 8,000	N/A	PW > CW

must be done to confirm this hypothesis, as well as to determine if there is any real benefit to the deeper penetration attained by pulsed lasers. Muscles such as the biceps and rectus femoris are not small organs, and have quite deep target tissue. Yet, various studies have shown significant improvement with CW lasers and CW LED. It remains to be seen whether or not pulsed lasers offer any additional advantage. Similarly, depression [20] and stroke studies [21] using LLLT have demonstrated that CW LED's and CW lasers (respectively) produce a beneficial therapeutic effect. There are reports from Anders' laboratory that fluences as low as 0.1–0.2 J/cm^2 may be optimal for cells in the brain [22]. However, further studies must be done to determine whether pulsed light, with higher peak power densities deeper into the brain tissues, might increase the effectiveness of these therapies.

STUDIES COMPARING CW AND PW

In this review thirty-three studies involving pulsed LLLT were examined. Of these studies, nine of them directly compared continuous wave (CW) with pulsed wave (PW) light, as recorded in Table 1. Six of these nine studies found PW to be more effective than CW. One study comparing CW and PW found both modes of operation to be equally effective, with no statistically significant difference between the two. Only two of the nine articles reported better results with CW than PW, although in both of these studies PW treated subjects were found to have better outcomes than placebo groups. One of the recurring limitations of the papers in this review was that like for like irradiation parameters were not used. For instance, Gigo-Benato et al. [23] found CW superior to PW in nerve regeneration, but is this because of the mode of operation (CW or PW) or because the CW laser used 808 nm and the pulsed laser used 905 nm?

TABLE 1.

Studies Comparing CW and PW

Of the six studies that found PW to be more effective than CW, four of them involved the use of LLLT to cure the following pathologies in vivo: wound healing, pain, and ischemic stroke. The two remaining studies reported pulsing to be beneficial in vitro; in the first such study, PW promoted bone stimulation more so than CW. The other in vitro study comparing CW and PW found the latter mode of operation better able to

penetrate through melanin filters, indicating that pulsing may be beneficial in reaching deep target tissue in dark-skinned patients.

In the wound healing study, Kymplova et al. [24] used a large sample size of women to study the effects of phototherapy on wound repair following surgical episiotomies (one of the most common surgical procedures in women). A pulsed laser emitted light (wavelength of 670 nm) at various frequencies (10, 25, and 50 Hz). The pulsed laser promoted wound repair and healing more so than the CW light source.

In the pain study, Sushko et al. [25] investigated the role of pulsed LLLT to attenuate pain in white male mice. The same wavelength of light was used as in Kymplova et al.'s study (670 nm), with the frequencies of 10, 600, and 8,000 Hz. Both modes of delivery (CW and PW) reduced the behavioral manifestations of somatic pain as compared to controls, but pulsed light (10 and 8,000 Hz in particular) was more effective.

The two studies involving pulsed LLLT and stroke were both done by Lapchak et al. [26]. Ischemic strokes were induced in rabbits, and a pulsed laser with a wavelength of 808 nm was used. In the first study, two frequencies of pulsed light were used (100 and 1,000 Hz), both of which reduced neurological deficits more so than CW. Accordingly, pulsed LLLT may play a major role in the management of stroke patients. Lapchak et al.'s second study attempted to prove the hypothesis that LLLT's neuroprotective effect following stroke was a result of enhanced mitochondrial energy production (increased ATP synthesis) [27]. As with the previous study, LLLT was administered following stroke induction. CW radiation raised cortical ATP levels but was unable to bring them back to baseline. PW radiation, on the other hand, not only mitigated the effects of stroke on cortical ATP levels, but was able to raise cortical ATP levels to higher than those found in healthy rabbits (those in which stroke was not induced). This study provides valuable insight into one of the potential cellular and molecular mechanisms behind the enhanced neurogenesis (and improved clinical outcomes) observed in subjects receiving transcranial LLLT following stroke.

One of the nine studies reviewed found CW and PW to be equally effective in the promotion of wound healing. This study compared the effects of a CW laser (632.8 nm) and a PW laser (904 nm) on the promotion of wound healing in rabbits. Both lasers improved tensile strength during wound healing, but did not significantly improve wound-healing rates. A combined laser (CW+PW) was also tested. All three of the laser regimens improved tensile strength to a similar extent.

As mentioned earlier, there were nine studies that compared CW and PW, only two of which found CW to be more effective. These two studies involved wound healing and nerve regeneration respectively. Al-Watban and Zhang [28] study involved rats that were inflicted with aseptic wounds. The rats were divided into three groups: a control group, those irradiated with continuous wave light, and those irradiated with pulsed light

at various repetition rates (100, 200, 300, 400, and 500 Hz). Of the pulse repetition rates administered, 100 Hz was the most efficacious and 500 Hz the least. Both CW and PW (635 nm) promoted wound healing, but CW was more efficacious. These results conflict with earlier studies that found pulsed light to be more beneficial in the promotion of wound healing. However, it should be noted that the difference between CW and PW treated subjects was small (a relative wound healing rate of 4.81 as compared to 4.32).

The second study that found CW to be more effective than PW involved nerve regeneration. There were three articles involving nerve regeneration, all of which found pulsed LLLT to be ineffective, as discussed in the section below entitled “Studies Involving Nerve Conduction and Regeneration.” Of these three, only Gigo-Benato et al. [23] compared CW (808 nm) and PW (905 nm). This study involved rats in which the left median nerve was completely transected and then repaired by end-to-end neurorrhaphy. The CW laser (808 nm) promoted faster nerve and muscle recovery than the pulsed laser (905 nm). However, Gigo-Benato also tested a combination of the CW and pulsed lasers, finding this to be the most effective of all. In other words, seven of the nine studies comparing CW and PW found pulsing to play a beneficial role. Only one of the nine studies found no role of PW, and even in this study the benefit of CW over PW was minimal.

STUDIES INVOLVING THE USE OF COMBINED LASERS (CW+PW)

We reviewed three studies, as recorded in Table 2, which investigated the role of a combined laser (using both CW and PW). Of these, only Gigo-Benato’s study compared the combined laser to stand alone CW or PW. This study has been discussed in the above section: the combined laser was found to be effective in stimulating nerve regeneration, more so than CW or PW alone.

TABLE 2.

Refs.	Subject	Condition	λ (nm)	*f* (Hz)	Other reported parameters	Results
Gigo-Benato et al. [23]	Rats	Nerve regeneration	808 (CW), 905 (PW), 808 + 905 (CW + PW)	10,000	Power: 416 mW (CW), 28 W (PW); energy density: 29 J/cm^2 (CW), 40 J/cm^2 (PW); pulse duration: 454 seconds (CW), 200 nanoseconds (PW)	Combined (CW + PW) > CW > PW
Branco and Naeser [62]	Humans	Pain	670 (CW), 904 (PW)	73, 584, 3,500	N/A	Combined regimen (CW + PW + TEN-TENS) effective
Naeser et al. [29]	Humans	Pain	632.8 (CW), 904 (PW)	73, 584, 3,500	Power: 15 mW (CW), 9.4 W (PW)	Combined regimen (CW + PW + TEN-TENS) effective

Studies Involving the Use of Combined Lasers (CW + PW)

The two other studies used a combined laser (CW and PW) to administer laser acupuncture, along with Transcutaneous Electrical Nerve Stimulation (TENS), to patients with symptoms of pain. Naeser et al. [29] administered this “triple therapy” to patients suffering from carpal tunnel syndrome (CTS). Eleven patients with mild-to-moderate symptoms of CTS were selected, all of who had failed to respond to standard medical or surgical treatment regimens. Subjects were divided into two groups, one of which received sham irradiation and the other that received a combined treatment of LLLT (CW and pulsed) and TENS. As compared to controls, the treated group experienced statistically significant improvement and remained stable for 1–3 years. The results of this study are promising, and indicate a possible role of LLLT and TENS in the conservative management of CTS.

Ceccherelli et al. [30] administered laser acupuncture to patients suffering from myofascial pain. In this double-blinded placebo controlled trial, patients received either the same “triple therapy” as in the Naeser et al. study (CW, PW, and TENS) or sham

irradiation, every other day over the course of 24 days. Results were encouraging, with the treatment group experiencing a significant improvement in symptoms, both immediately after the treatment regimen and at a 3-month follow up visit.

Refs.	Subject	Condition	λ (nm)	*f* (Hz)	Other reported parameters	Results
Schubert [63]	Humans	Wound healing	637, 956	8.58, 15.6, 31.2, 78, 287, and 702	Irradiance: 21 W/m^2, 55 W/m^2; duty cycle: 80%	PW > untreated controls
Rezvani et al. [35]	Pigs	Prevention of post-radiation complication of dermal necrosis	660, 820, 880, 950	2.5 and 5,000	Power: 15 mW; power density: 120 mW/cm^2; energy density: 0.22, 0.54, 1.08, 2.16, 4.32, and 10.8 J/cm^2; duty cycle: 80%; pulse duration: 320 milliseconds with 80 milliseconds-pause between pulses	PW > untreated controls; 5 kHz most effective
Bagis et al. [64]	Rats	Nerve regeneration	904	4, 8, 16, 32, 64, and 128	Power: 27 W; energy density: 0.005–2.5 J/cm^2; pulse duration: 220 nanoseconds	PW made no statistically significant effect as compared to untreated controls
Chen et al. [41]	Rats	Nerve regeneration	904	5,000 and 20,000	Energy density: 2.23–3.88 J/cm^2 (5 kHz), 8.92–15.5 J/cm^2 (20 kHz)	PW decreased (as opposed to increased) nerve regeneration as compared to sham-irradiated controls

Refs.	Subject	Condition	λ (nm)	*f* (Hz)	Other reported parameters	Results
Walsh et al. [38]	Humans	Nerve conduction	820	9.12 and 73	Power: 50 mW; energy density: 9.55 J/cm^2; duty cycle: 80%	PW made no statistically significant effect as compared to untreated controls
Bagis et al. [39]	Frogs	Nerve conduction	904	4, 8, 16, 64, and 128	Power: 27 W; energy density: 0.005–2.5 J/cm^2; pulse duration: 220 nanoseconds	PW made no statistically significant effect as compared to untreated controls
Comelekoglu et al. [40]	Frogs	Nerve conduction	904	1, 4, 16, and 64	Power: 27 W; energy density: 0.005–2.5 J/cm^2; pulse duration: 220 nanoseconds	PW made no statistically significant effect as compared to untreated controls
Longo et al. [31]	Rats	Wound healing	904	1,500 and 3,000	Power: 20 W; power density: 5 mw/cm^2 (1.5 kHz), 10 mw/cm^2 (3 kHz); energy density: 3 J; pulse duration: 200 nanoseconds	PW > untreated controls; only 3,000 Hz was effective in promoting wound healing
Korolev and Zagorskaia [32]	Rats	Wound healing	850–910	500 and 3,000	N/A	PW > untreated controls; 500 Hz more effective

Refs.	Subject	Condition	λ (nm)	*f* (Hz)	Other reported parameters	Results
Vasheghani et al. [65]	Rats	Wound healing	890	80	Power: 75 W; energy density: 0.396 J/cm^2; pulse duration: 180 microseconds	PW > untreated controls
Hopkins et al. [66]	Humans	Wound healing	820	700	Energy density: 8 J/cm^2	PW > untreated controls
Kucerova et al. [67]	Humans	Wound healing	670, 632.8	5, 292, and 9,000	Power: 20 mW; energy density: 1.5 J/cm^2	PW > untreated controls
el Sayed and Dyson [33]	Rats	Wound healing	820	2.5, 20, 292, and 20,000	Power: 800 mW/cm^2; energy density: 21.6 J km; pulsing duration: 360, 45, 3, and 0.045 milliseconds for 2.5, 20, 292, and 20,000 Hz respectively; duty cycle: 90%	PW > untreated controls; 20 and 292 Hz most effective
Morrone et al. [68]	In vitro (chondrocytes from humans)	Cartilage growth		100, 300, and 500	Power: 1W; energy: 300 J	PW > untreated controls
Ponnudurai et al. [34]	Rats	Pain	632.8	4, 60, and 200	N/A	PW > untreated controls; 4Hz acted most rapidly but

Refs.	Subject	Condition	λ (nm)	*f* (Hz)	Other reported parameters	Results
						transiently; 60 Hz had delayed but longer lasting effect; 200 Hz had no effect
Mokhtar et al. [69]	Humans	Pain	660–950	16 and 73	Power: 532 mW; pulse duration: 50 milliseconds with 12.5 milliseconds period between pulses; energy: 383 J; duty cycle: 80%	PW > untreated controls (significant but very minimal hypoalgesic effect was found)
Miriutova et al. [70]	Humans	Pain	850–910	N/A	N/A	PW > untreated controls
Craig et al. [42]	Humans	Pain	660–950	2.5, 5, and 20	Total average radiant exposure: 31.7 J/cm^2	PW made no statistically significant effect as compared to untreated controls
de Bie et al. [43]	Humans	Pain	904	500 and 5,000	Power: 25 W; energy density: 0.5 and 5 J/cm^2; pulse duration: 200 nanoseconds	PW made no statistically significant effect as compared to untreated controls

Refs.	Subject	Condition	λ (nm)	*f* (Hz)	Other reported parameters	Results
Ceccherelli et al. [30]	Humans	Pain	N/A	N/A	N/A	PW > untreated controls
Read et al. [71]	Human	Brain damage (causing hyperphagia and pica)	950	2.5 and 10	Power: 25 mW	PW > untreated control; significant but transient reduction of symptoms

In both preceding studies, the combined regimen of CW, PW, and TENS was compared to untreated controls, and found to be effective. However, neither study compared CW and PW or administered CW, PW, or TENS individually. As such, it is difficult to determine whether standalone CW or PW would have produced similar results, or if the combined regimen (along with TENS) was necessary.

STUDIES EVALUATING THE USE OF PULSED LASERS

Of the 33 studies reviewed, 21 of them compared PW treated subjects with untreated controls, as reported in Table 3. Of these, fourteen studies found pulsed LLLT to be effective, whereas seven of them found PW treated subjects to have no benefit over untreated controls. Only one study found PW to have a worse outcome than controls. Of the fourteen studies that found pulsed LLLT to be effective, seven involved the promotion of wound healing, four involved the attenuation of pain, two involved the promotion of bone and cartilage growth respectively, and one involved the treatment of a very rare condition (hyperphagic syndrome caused by traumatic brain injury). Of the seven studies that found no benefit to pulsed light, three involved the promotion of nerve conduction, two involved the promotion of nerve regeneration, and the remaining two involved the attenuation of pain.

TABLE 3.

Studies Evaluating the Use of Pulsed Lasers

Studies Comparing Various Pulse Repetition Rates

If pulsed LLLT is effective (or ineffective), then what pulse repetition rates are to be used (or avoided)? Ten of the 33 articles reviewed tested and compared various

Refs.	Subject	Condition	λ (nm)	*f* (Hz)	Other reported parameters	Results
Rezvani et al. [35]	Pigs	Prevention of post-radiation complication of dermal necrosis	660, 820, 880, 950	2.5 and 5,000	Power: 15 mW; power density: 120 mW/cm^2; energy density: 0.22, 0.54, 1.08, 2.16, 4.32, and 10.8 J/cm^2; duty cycle: 80%; pulse duration: 320 milliseconds with 80 milliseconds-pause between pulses	PW > untreated controls; 5 kHz most effective
Brondon et al. [36]	In vitro (human HEP-2 cells)	Increasing the penetration depth of light through melanin filters	670	6, 18, 36, 100, and 600	Power: 10 mW; energy density: 5 J/cm^2	PW > CW; 100 and 600 Hz most effective
Lapchak et al. [26]	Rabbits	Ischemic stroke	808	100 and 1,000	Power density: 7.5 mW/cm^2; ON time: 0.3 milliseconds (1,000 Hz), 2 milliseconds (100 Hz); average energy delivered to the brain: 0.9–1.2 J; duty cycle: 30% and 20%	PW > CW; both 1 kHz and 100 Hz had similar effect with no significant difference between the two
Longo et al. [31]	Rats	Wound healing	904	1,500 and 3,000	Power: 20 W; power density: 5 mw/cm^2 (1.5 kHz), 10 mw/cm^2 (3 kHz); energy density: 3 J; pulse duration: 200 nanoseconds	PW > untreated controls; only 3,000 Hz was effective in promoting wound healing
Korolev and Zagorskaia [32]	Rats	Wound healing	850–910	500 and 3,000	N/A	PW > untreated controls; 500 Hz more effective
Al-Watban and Zhang [28]	Rats	Wound healing	635	100, 200, 300, 400, and 500	Power density: 0.89 mW/cm^2; energy density: 1.0 J/cm^2	CW > PW; of the pulsed frequencies used, 100 Hz was most effective (but less so than CW)

Refs.	Subject	Condition	λ (nm)	*f* (Hz)	Other reported parameters	Results
el Sayed and Dyson [33]	Rats	Wound healing	820	2.5, 20, 292, and 20,000	Power: 800 mW/cm^2; Energy density: 21.6 J km; pulsing duration: 360, 45, 3, and 0.045 milliseconds for 2.5, 20, 292, and 20,000 Hz respectively; duty cycle: 90%	PW > untreated controls; 20 and 292 Hz most effective
Ueda and Shimizu [37]	In vitro (osteoblast-like cells from fetal rat calvariae)	Bone stimulation	830	1, 2, and 8 Hz	N/A	PW > CW; 2 Hz most effective
Ponnudurai et al. [34]	Rats	Pain	632.8	4, 60, and 200	N/A	PW > untreated controls; 4 Hz acted most rapidly but transiently; 60 Hz had delayed but longer lasting effect; 200 Hz had no effect
Sushko et al. [25]	Mice	Pain	610–670, 850–910	10, 600, and 8,000	N/A	PW > CW; 10 and 8,000 Hz most effective

repetition rates, as reported in Table 4. Four of these studies involved the use of pulsed LLLT to promote wound healing. Longo et al. [31] used the pulse repetition rates of 1,500 and 3,000 Hz, and found only the latter setting to promote wound healing. Korolev et al. [32] similarly used two pulse repetition rates, 500 and 3,000 Hz. In this case, both were found to be effective but 500 Hz was more so. Al-Watban and Zhang [28] compared five different pulse repetition rates (100, 200, 300, 400 and 500 Hz), finding 100 Hz to be the most effective and 500 Hz the least. el Sayed and Dyson [33] compared four different pulse repetition rates (2.5, 20, 292, and 20,000 Hz), and found only the two middle values (20 and 292 Hz) beneficial. The more effective pulse repetition rates in these four studies were very disparate, including 20, 100, 292, 500, and 3,000 Hz (a range of 20–3,000 Hz).

TABLE 4.

Studies Comparing Various Pulse Repetition Rates

Two studies compared the role of various pulse repetition rates in the attenuation of pain. Ponnudurai et al. [34] used laser photobiostimulation to decrease pain levels in rats, and investigated the effect of using various pulsing frequencies (4, 60, and 200 Hz). The rat tail-flick test was utilized, and tail-flick latencies were measured at five intervals between 30 minutes and 7 days following irradiation. The pulsing frequency of 4 Hz increased pain threshold rapidly but very transiently, whereas 60 Hz produced a delayed but longer lasting effect. On the other hand, 200 Hz failed to produce any hypoalgesic effect whatsoever. Sushko et al. [25] conducted a similar experiment, using mice instead of rats. The center of pain was irradiated (610–910 nm) for 10 minutes with either CW or pulsed light (10, 600, and 8,000 Hz). Both modes of delivery (CW and pulsed) reduced the behavioral manifestations of somatic pain as compared to controls, but pulsed light was more effective. In particular, 10 and 8,000 Hz produced the best effect. The more effective pulse repetition rates from these two studies (involving pain attenuation) included 4, 10, 60, and 8,000 Hz (a range of 4–8,000 Hz), and the less effective pulse repetition rates included 200 and 600 Hz.

Lapchak et al. [26] not only compared CW and PW, but also pulsed light at two different repetition rates, P1 (1,000 Hz) and P2 (100 Hz). Ischemic strokes were induced in rabbits, and the neuroprotective effects of LLLT were assessed via behavioral analysis 48 hours post-stroke. Both P1 (1,000 Hz) and P2 (100 Hz) produced a similar effect (superior to CW).

Rezvani et al. [35] studied the use of low level light therapy to prevent X-ray induced late dermal necrosis. An X-ray dose of 23.4 Gy is known to invariably cause dermal necrosis after 10–16 weeks. This dose was delivered to pigs, which were then treated with LLLT for several weeks using various wavelengths (660, 820, 880, and 950 nm) pulsed at either 2.5 or 5,000 Hz. Light pulsed at 2.5 Hz did not reduce the incidence of dermal necrosis. On the other hand, light pulsed at 5,000 Hz significantly reduced (P = 0.001) the incidence to 52% when given 6–16 weeks after irradiation.

Of the 10 articles reviewed that compared various pulse repetition rates, two of them involved in vitro experiments. Brondon et al. [36] undertook a study to determine if pulsing light would overcome the filtering effects of melanin. Melanin filters were placed in front of human HEP-2 cells, which were then irradiated for 72 hours (670 nm wavelength) with either CW or pulsed light at various repetition rates (6, 18, 36, 100, and 600 Hz). Both cell proliferation and oxidative burst activity, were increased in the group treated with pulsed light, indicating that pulsed light is indeed better able to penetrate melanin rich skin. Specifically, cell proliferation was maximal at 100 Hz at 48

Refs.	Subject	CW	PW	λ (nm)	*f* (Hz)	Other reported parameters	Results
Schubert [63]	Humans		X	637, 956	8.58, 15.6, 31.2, 78, 287, and 702	Irradiance: 21 and 55 W/m^2; duty cycle: 80%	PW > untreated controls
Kymplova et al. [24]	Humans	X	X	670	10, 25, and 50	Power: 20 mW; energy density: 2 J/cm^2	PW > CW
Longo et al. [31]	Rats		X	904	1,500 and 3,000	Power: 20 W; power density: 5 mw/cm^2 (1.5 kHz), 10 mw/cm^2 (3 kHz); energy density: 3 J; pulse duration: 200 nanoseconds	PW > untreated controls; only 3,000 Hz was effective in promoting wound healing
Braverman et al. [61]	Rabbits	X	X	632.8 (CW), 904 (PW)	4,672	Power: 10 mW (CW), 50 mW (PW); energy density: 1.65 J/cm^2 (CW), 8.25 J/cm^2 (PW); pulse duration: 200 nanoseconds	Both CW and PW improved tensile strength, and no statistically significant difference between the two
Korolev and Zagorskaia [32]	Rats		X	850–910	500 and 3,000	N/A	PW > untreated controls; 500 Hz more effective
Al-Watban and Zhang [28]	Rats		X	635	100, 200, 300, 400, and 500	Power density: 0.89 mW/cm^2; energy density: 1.0 J/cm^2	CW > PW; of the pulsed frequencies used, 100 Hz was most effective (but less so than CW)
Vasheghani et al. [65]	Rats	X	X	890	80	Power: 75 W; energy density: 0.396 J/cm^2; pulse duration=180 microseconds	PW > untreated controls
Hopkins et al. [66]	Humans		X	820	700	Energy density: 8 J/cm^2	PW > untreated controls
Kucerova et al. [67]	Humans		X	670, 632.8	5, 292, and 9,000	Power: 20 mW; energy density: 1.5 J/cm^2	PW > untreated controls

Refs.	Subject	CW	PW	λ (nm)	*f* (Hz)	Other reported parameters	Results
el Sayed and Dyson [33]	Rats		X	820	2.5, 20, 292, and 20,000	Power: 800 mW/cm; energy density: 21.6 J km; pulsing duration: 360, 45, 3, and 0.045 milliseconds for 2.5, 20, 292, and 20,000 Hz respectively; duty cycle: 90%	PW > untreated controls; 20 and 292 Hz most effective

and 72 hours (n = 4, P≤0.05), and oxidative burst was maximal at 600 Hz (n = 4, P≤0.05).

Ueda and Shimizu [37] studied the effects of pulsed low-level light on bone formation in vitro. Osteoblast-like cells were isolated from fetal rat calvariae; one group was not irradiated at all, another was irradiated with continuous wave light, and the third group with pulsed light at three repetition rates (1, 2, and 8 Hz). As compared to the control group, both CW and PW light resulted in increased cellular proliferation, bone nodule formation, alkaline phosphatase (ALP) gene expression, and ALP activity. Pulsed light at 2 Hz stimulated these factors the most.

Out of all 10 articles that compared various pulse repetition rates, the following pulse repetition rates were found to be beneficial: 2, 10, 20, 100, 292, 500, 600, 1,000, 3,000, 5,000, and 8,000 Hz. In this wide range of frequencies (2–8,000 Hz), no particular frequencies stood out as being particularly more or less useful than others.

STUDIED INVOLVING WOUND HEALING

Ten studies out of the 33 involved LLLT's role in the promotion of wound healing, as recorded in Table 5. Only two of these studies compared CW and PW. Kymplova et al. [24] found pulsed LLLT to promote wound healing over CW, whereas Al-Watban and Zhang [28] found CW to be slightly more effective than PW. Both studies used light of a similar wavelength (670 vs. 635 nm), although the pulse repetition rates used by Kymplova et al. were lower (10–50 Hz vs. 100–500 Hz in Al-Watban et al.'s study). The energy densities applied were also different (2 J/cm^2 vs. 1 J/cm^2).

TABLE 5.
Studied Involving Wound Healing

Refs.	Subject	Condition	CW	PW	λ (nm)	*f* (Hz)	Other reported parameters	Results
Gigo-Benato et al. [23]	Rats	Nerve regeneration	X	X	808 (CW), 905 (PW), 808 + 905 (CW + PW)	10,000	Power: 416 mW (CW), 28 W (PW); energy density: 29 J/cm^2 (CW), 40 J/cm^2 (PW); pulse duration: 454 seconds (CW), 200 nanoseconds (PW)	Combined (CW + PW) > CW > PW
Bagis et al. [64]	Rats	Nerve regeneration		X	904	4. 8, 16, 32, 64, and 128	Power: 27 W; energy density: 0.005–2.5 J/cm^2; pulse duration: 220 nanoseconds	PW made no statistically significant effect as compared to untreated controls
Chen et al. [41]	Rats	Nerve regeneration		X	904	5,000 and 20,000	Energy density: 2.23–3.88 J/cm^2 (5 kHz), 8.92–15.5 J/cm^2 (20 kHz)	PW decreased (as opposed to increased) nerve regeneration as compared to sham-irradiated controls
Walsh et al. [38]	Humans	Nerve conduction		X	820	9.12 and 73	Power: 50 mW; energy density: 9.55 J/cm^2; duty cycle: 80%	PW made no statistically significant effect as compared to untreated controls
Bagis et al. [39]	Frogs	Nerve conduction		X	904	4, 8, 16, 64, and 128	Power: 27 W; energy density: 0.005–2.5 J/cm^2; pulse duration: 220 nanoseconds	PW made no statistically significant effect as compared to untreated controls

Refs.	Subject	Condition	CW	PW	λ (nm)	*f* (Hz)	Other reported parameters	Results
Comelekoglu et al. [40]	Frogs	Nerve conduction		X	904	1, 4, 16, and 64	Power: 27 W; energy density: 0.005–2.5 J/cm^2; pulse duration: 220 nanoseconds	PW made no statistically significant effect as compared to untreated controls

Every study reviewed found pulsed LLLT effective in promoting wound healing (as compared to untreated controls), including the Al-Watban et al. study. Six of these studies used light in the wavelength range of 820–956 nm, and four in the range of 632.8–670 nm. Once again, a wide range of frequencies were used (2.5–20,000 Hz), most of which were found to promote wound healing. (Tested frequencies included 2.5, 5, 8.58, 10, 15.6, 20, 25, 31.2, 50, 78, 80, 287, 292, 500, 700, 3,000, 4,672, 9,000, and 20,000 Hz). Most of these articles also reported energy densities, usually in the range of 1–2 J/cm^2.

STUDIES INVOLVING NERVE CONDUCTION AND REGENERATION

We reviewed three articles evaluating the role of pulsed LLLT in the promotion of nerve conduction, and another three involving nerve regeneration, as reported in Table 6. Unlike the studies involving wound healing where positive outcomes were reported, all six of these studies reported negative outcomes with pulsed light. Five of these studies found PW to have no statistically significant effect on outcome, whereas one of them found PW to have a deleterious effect. There was no study that directly compared CW and PW in regards to nerve conduction. Walsh et al. [38] conducted a study with 32 human volunteers to determine if pulsed LLLT would influence nerve conduction in the superficial radial nerve. Action potentials were measured pre- and post-irradiation (at 5, 10, and 15 minutes). No significant difference was appreciated between control and treatment groups, indicating that LLLT with those particular pulsing parameters and dosimetry had no specific neurophysiologic effects on nerve conduction. Bagis et al. [39] and Comelekoglu et al. [40] obtained similar negative results using frog nerves. Walsh et al. used a wavelength of 820 nm, whereas Bagis et al. used a 904 nm laser. All three studies tested pulse repetition rates within the range of 1–128 Hz.

TABLE 6.
Studies Involving Nerve Conduction and Regeneration

Refs.	Subject	CW	PW	λ (nm)	f (Hz)	Other reported parameters	Results
Ponnudurai et al. [34]	Rats		X	632.8	4, 60, and 200	N/A	PW > untreated controls; 4 Hz acted most rapidly but transiently; 60 Hz had delayed but longer lasting effect; 200 Hz had no effect
Mokhtar et al.[69]	Humans		X	660–950	16 and 73	Power: 532 mW; pulse duration: 50 milliseconds with 12.5 milliseconds period between pulses; energy: 383 J; duty cycle: 80%	PW > untreated controls
Miriutova et al.[70]	Humans		X	850–910		N/A	PW > untreated controls
Craig et al.[42]	Humans		X	660–950	2.5, 5, and 20	Total average radiant exposure: 31.7 J/cm^2	PW made no statistically significant effect as compared to untreated controls
Sushko et al.[25]	Mice	X	X	610–670, 850–910	10, 600, and 8,000	N/A	PW > CW; 10 and 8,000 Hz most effective
de Bie et al.[43]	Humans		X	904	500 and 5,000	Power: 25 W; energy density: 0.5 and 5 J/cm^2; pulse duration: 200 nanoseconds	PW made no statistically significant effect as compared to untreated controls
Ceccherelli et al.[30]	Humans		X	N/A	N/A	N/A	PW > untreated controls
Branco and Naeser [62]	Humans	X	X	670 (CW), 904 (PW)	73, 584, 3,500	N/A	Combined regimen (CW, pulsed, and TENS) improved outcome as compared to untreated controls

Refs.	Subject	CW	PW	λ (nm)	f (Hz)	Other reported parameters	Results
							(CW and PW not administered individually in this study)
Naeser et al. [29]	Humans	X	X	632.8 (CW), 904 (PW)	73, 584, 3,500	Power: 15 mW (CW), 9.4 W (PW)	Combined regimen (CW, PW, and TENS) improved outcome as compared to untreated controls (CW and PW were not administered individually in this study)

Similarly, the nerve regeneration studies reviewed reported negative outcomes. Chen et al. [41] found PW to have a counterproductive effect, reducing nerve regeneration as compared to untreated controls. Only one study compared CW with PW, and found the former to be superior to the latter. However, the combined laser (CW+PW) was superior to CW alone, indicating that there might in fact be a role of pulsing in nerve regeneration.

STUDIES INVOLVING PAIN ATTENUATION

Nine of the thirty-three studies involved pulsed LLLT's role in the attenuation of pain, as reported in Table 7. Of these, only one of them directly compared CW and PW. This study was conducted by Sushko et al. [25] and found that although both CW and PW decreased pain levels, PW was more effective. This study also determined that pulse repetition rates of 10 and 8,000 Hz were more effective than 600 Hz. Ponnudurai et al. [34] similarly compared various pulse repetition rates (4, 60, and 200 Hz). A rapid but transient analgesic effect was exhibited with 4 Hz, whereas a delayed but longer lasting effect was achieved with 60 Hz. On the other hand, 200 Hz failed to produce any analgesic effect whatsoever.

TABLE 7.
Studies Involving Pain Attenuation

Two of the studies used a combined laser (CW+PW) along with TENS; both found the combined regimen to be effective. The five remaining studies compared pulsed LLLT with untreated controls. Three of these studies found pulsed LLLT to be effective, whereas two did not. Of the nine total studies on pain attenuation, seven found pulsed LLLT to be effective in its role of attenuating pain. Only two studies found no statistically

significant effect. However, it should be noted that both of these involved pain of a different nature than commonly tested in pulsed LLLT studies. The first of these was by Craig et al. [42] and involved the use of pulsed LLLT to relieve the symptoms of delayed-onset muscle soreness (DOMS). DOMS refers to the feeling of pain and muscle stiffness that can result 1–3 days after intense sporting activity such as weightlifting. This pain is duller in quality than that tested in the other studies. The second study that showed no benefit to pulsed LLLT, published by de Bie et al. [43], involved the treatment of lateral ankle sprains.

STUDIES INVOLVING ISCHEMIC STROKE

Table 8 records the two studies that involved pulsed LLLT and stroke. In the first study, PW but not CW decreased neurological deficits when delivered six hours post-stroke. Two pulse repetition rates were tested (100 and 1,000 Hz) and found to be equally effective. On the other hand, both CW and PW produced no benefit if delivered 12 hours post-stroke, indicating that timely administration of LLLT is essential.

TABLE 8.

Refs.	Subject	Condition	CW	PW	λ (nm)	*f* (Hz)	Other reported parameters	Results
Lapchak et al. [26]	Rabbits	Ischemic stroke	X	X	808	100 and 1,000	Power density: 7.5 mW/cm^2; on time: 0.3 milliseconds (1,000 Hz), 2 milliseconds (100 Hz); average energy delivered to the brain: 0.9–1.2 J; duty cycle: 30% and 20%	PW > CW; both 1 kHz and 100 Hz had similar effect with no significant difference between the two
Lapchak and De Taboada [27]	Rabbits	Ischemic stroke	X	X	808	100	Cortical irradiance: 7.5 mW/cm^2 (CW), 37.5 mW/cm^2 (PW); cortical fluence: 0.9 J/cm^2 (CW), 4.5 J/cm^2 (PW)	PW > CW

Studies Involving Stroke

The second study investigated the possible mechanisms behind the neuroprotective effect of LLLT. It was postulated that LLLT enhances mitochondrial energy production (and ATP synthesis), which allows for enhanced neurogenesis. This hypothesis was tested using the rabbit small clot embolic stroke model (RSCEM). Four groups of rabbits were used: (1) a naïve control group which was neither embolized or irradiated, (2) a placebo group which was embolized and sham irradiated, (3) an embolized group which

was irradiated with CW (808 nm), and (4) an embolized group which was irradiated with pulsed light (808 nm) at two different frequencies. Forty-five percent less cortical ATP was measured in the second group (placebo) as compared to the first (naïve), confirming the hypothesis that ischemic strokes decrease cortical mitochondrial energy. All laser irradiated groups were able to mitigate this effect. CW radiation managed to raise the cortical ATP levels by 41%, whereas PW administration raised these levels by over 150%. Surprisingly, this was even higher than the cortical ATP content measured in naïve rabbits that had never suffered stroke.

OTHER APPLICATIONS OF PULSED MODALITIES IN BIOMEDICINE

Many of the modalities of treatment employed in biomedicine and physical therapy are used in pulsed format [44]. Electricity, electromagnetic fields and ultrasound are applied with particular pulse structures. It may be possible to gain some insight into the effect of pulsing structures in LLLT by a brief review of the other pulsed modalities. Transcutaneous electrical neural stimulation (TENS) is the application of pulses of electric current to the skin [45]. This application stimulates the brain and has been used for the treatment of various psychological and neurological conditions, including Parkinson's, epilepsy, chronic pain, depression, and neuromuscular rehabilitation. Frequencies usually fall between 5 and 25 Hz, but may range from 2 to 80 Hz [46]. Deep brain stimulation (DBS) is a surgical treatment involving the implantation of a brain pacemaker, a medical device that sends electrical impulses to specific parts of the brain. DBS has the potential to provide substantial benefit to patients suffering from a variety of neurological conditions, including epilepsy, Parkinson's disease, dystonia, Tourette's syndrome, and depression [47]. The Food and Drug Administration (FDA) approved DBS at 130 Hz as a treatment for essential tremor in 1997, for Parkinson's disease in 2002, and dystonia in 2003. Pulsed electromagnetic field (PEMF) therapy has been used for a wide range of conditions, including bone healing and regeneration [48], osteoporosis [49], arthritis [50] wound healing and pain [51], carpal tunnel syndrome [52], spinal cord injury [53], nerve regeneration [54], soft tissue injuries [55], and cancer [56]. Frequencies used for these conditions range from 1 Hz ("low") to 200 Hz ("high"). Transcranial magnetic stimulation (TMS) is a noninvasive method used to excite neurons in the brain. Weak electric currents are induced by butterfly coils positioned above the head. TMS has been approved for the treatment of resistant depression in several countries and is under investigation for migraine [57], aphasia [58], and tinnitus [59]. Low-intensity pulsed ultrasound (LIPUS) utilizes a non-thermal mechanism of action, which can be used to promote bone healing by inducing the expression of growth factors and prostaglandins, which stimulate osteoblasts, chondrocytes and fibroblasts [60].

CONCLUSION

There has been remarkably little information available in the peer-reviewed literature on the rationale for using pulsed lasers or pulsed light in LLLT rather than CW. Moreover

there is no consensus on the effects of different frequencies and pulse parameters on the physiology and therapeutic response of the various disease states that are often treated with laser therapy. This has allowed manufacturers to claim advantages of pulsing without hard evidence to back up their claims.

CW light is the gold standard and has been used for all LLLT applications. However, this review of the literature indicates that overall pulsed light may be superior to CW light with everything else being equal. This seemed to be particularly true for wound healing and post-stroke management. On the other hand, PW as a solo treatment may be less beneficial than CW in patients requiring nerve regeneration. This could possibly be explained by the mechanism of action LLLT that can either cause cell stimulation or cell inhibition or both stimulation and inhibition at the same time on different cell types. It is possible that stimulation in neurons is desired to promote neurogenesis following stroke (increased mitochondrial synthesis of ATP results in more energy for neurons to regenerate themselves), whereas inhibition of inflammatory cells, inhibition of immune response or inhibition of the glial scar may also occur at the same time. The logic in favor of PW is that cells may need periods of rest, without which they can no longer be stimulated further.

Considering that the biology of LLLT is known to be complex, it is likely that there may several optimal sets of pulse parameters and that these may relate to the specific wavelengths and chromophores and may well also be affected by other optical properties of tissues.

It was impossible to draw any meaningful correlations between pulse frequency and pathological condition, due to the wide-ranging and disparate data. As for other pulse parameters, these were in general poorly and inconsistently reported. It is suggested that future researchers consistently report the following parameters: wavelength, frequency, duty cycle, peak power, average power, peak power density, average power density, and energy density. Careful reporting of these values would make it easier for future reviewers to find useful patterns. Furthermore, a concerted effort should be made to use like-for-like parameters when comparing CW and pulsed lasers. This limitation notwithstanding, this review indicates that pulsing will continue to play an important role in LLLT especially for applications where deep tissue penetration is required. Nevertheless, much more research remains to be done at both basic science and clinical levels before the role of pulsing in LLLT becomes clear and completely understood.

Acknowledgments

Contract grant sponsor: NIH; Contract grant number: R01AI050875; Contract grant sponsor: Center for Integration of Medicine and Innovative Technology; Contract grant number: DAMD17-02-2-0006; Contract grant sponsor: CDMRP Program in TBI;

Contract grant number: W81XWH-09-1-0514; Contract grant sponsor: Air Force Office of Scientific Research; Contract grant number: FA9950-04-1-0079.

The Use of Low Level Laser Therapy (LLLT) For Musculoskeletal Pain

Howard B Cotler [1,2,*], Roberta T Chow [3], Michael R Hamblin [4,5,6], James Carroll [7]
PMCID: PMC4743666 NIHMSID: NIHMS716157 PMID: 26858986
The publisher's version of this article is available at MOJ Orthop Rheumatol

Abstract

Pain is the most common reason for physician consultation in the United States. One out of three Americans is affected by chronic pain annually. The number one reason for missed work or school days is musculoskeletal pain. Currently accepted therapies consist of non-steroidal anti-inflammatory drugs, steroid injections, opiate pain medications and surgery, each of which carries their own specific risk profiles. What is needed are effective treatments for pain which have an acceptably low risk-profile. For over forty years, low level laser (light) therapy (LLLT) and LED (light emitting diode) therapy (also known as photobiomodulation) has been shown to reduce inflammation and edema, induce analgesia, and promote healing in a range of musculoskeletal pathologies. The purpose of this paper is to review the use of LLLT for pain, the biochemical mechanisms of action, the dose response curves, and how LLLT may be employed by orthopedic surgeons to improve outcomes and reduce adverse events.

With the predicted epidemic of chronic pain in developed countries, it is imperative to validate cost-effective and safe techniques for managing painful conditions which would allow people to live active and productive lives. Moreover the acceptance of LLLT (which is currently being used by many specialties around the world) into the armamentarium of the American health care provider would allow for additional treatment options for patients. A new cost-effective therapy for pain could elevate quality of life while reducing financial strains.
Keywords: Musculoskeletal, Pain, Low level laser therapy, Photobiomodulation, Injury repair

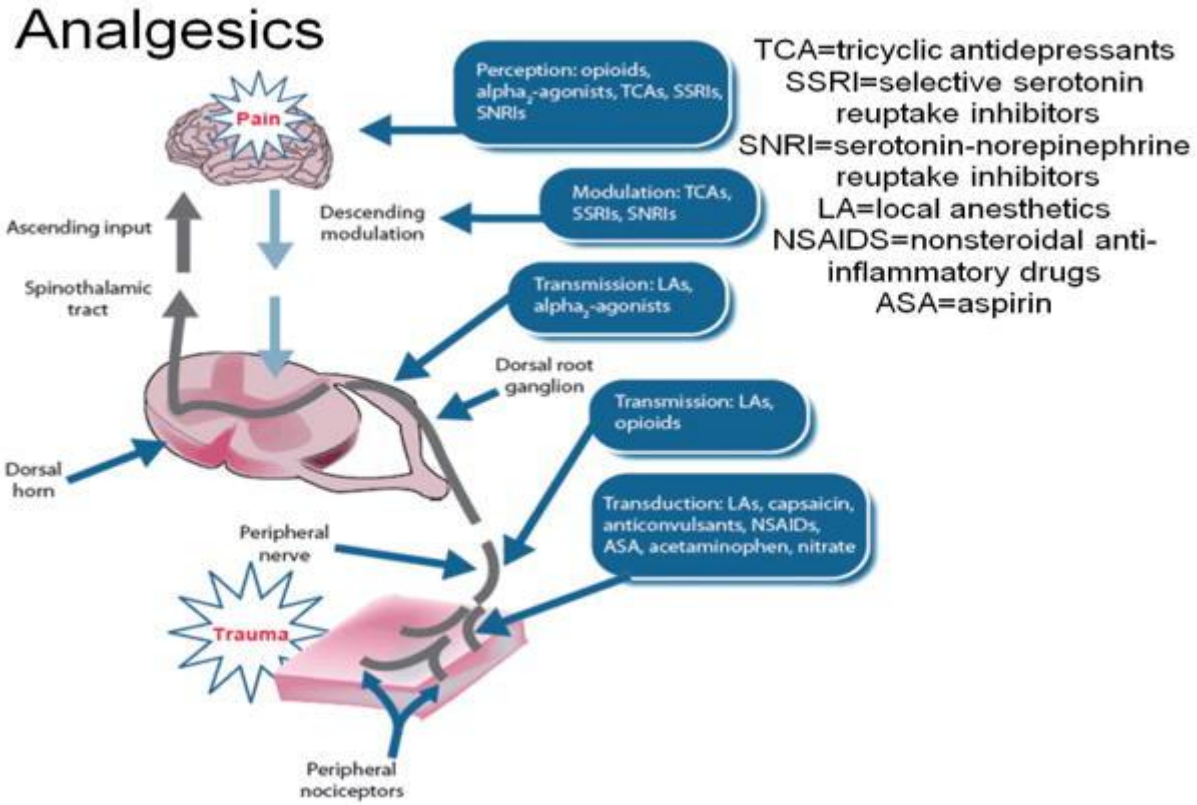
Introduction

Musculoskeletal pain affects 116 million Americans annually at a cost of $635 billion a year in medical bills, lost productivity and missed work or school [1,2]. All therapeutic treatments have their benefits, but also possess different side effects, risks and or complications. The current treatment for musculoskeletal pain includes modalities, immobilization, medications, chiropractic care, physical therapy, behavioral management, injections and/or surgery. These standard therapies have their particular associated risks/side effect profiles including peptic ulcers/gastric bleeding [3], systemic

effects (cardiovascular) [4], infections (including epidural abscess) [5], narcotic dependency/addiction [6], deformities, neurologic deficits, and surgical complications [7]. The natural history of chronic pain is one of increasing dysfunction, impairment and possible disability.

The definition of pain by the “International Association for the Study of Pain” states: “Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage [8]”. Withdrawal of the painful stimulus usually resolves pain promptly. Sometimes however, pain persists in spite of removal of the stimulus and even after healing of the body. Pain can also arise in the absence of any stimulus, disease or injury. Acute pain is considered to last less than thirty days, while chronic pain is of more than six months duration or as “pain that extends beyond the expected period of healing”. There are three different types of pain; nociceptive, neuropathic and central. The current medical treatment of pain or analgesics is directed at various steps of the pain pathways (Figure 1). Clinically, low level laser therapy (LLLT) can treat nociceptive [9] and neuropathic pain [10], while central pain has not yet been proven to be responsive to LLLT.

Figure 1.



Site of analgesic action on the pain pathway.

What is LLLT?

Low Level Laser Therapy (LLLT) sometimes known as Low Level Light Therapy or Photobiomodulation (PBM) is a low intensity light therapy. The effect is photochemical not thermal. The light triggers biochemical changes within cells and can be compared to the process of photosynthesis in plants, where the photons are absorbed by cellular photoreceptors and triggers chemical changes.

History of LLLT

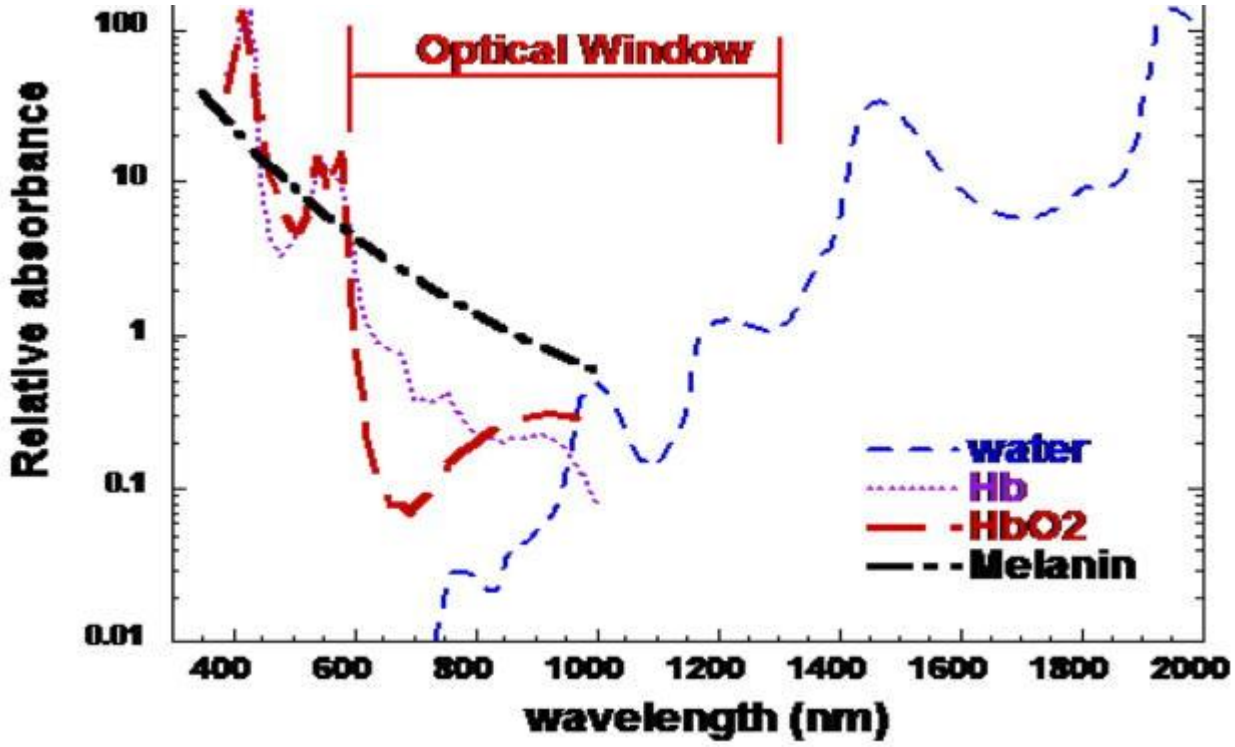
In 1903, Dr. Nils Finsen was awarded a Nobel Prize for his contribution to the treatment of diseases, especially lupus vulgaris, with concentrated light radiation [11]. In 1960, Professor Maiman TH [12] built the first working red ruby laser [12], but it was not until 1967 when Mester E et al. [13,14] was able to demonstrate the phenomenon of “laser bio stimulation” [13,14]. In 1999, Whelan H et al. [15] presented his work on the medical applications of light emitting diodes (LED) for use on the NASA space station [15]. Subsequently over 400 Phase III randomized, double-blind, placebo-controlled trials have been published, with over 4000 laboratory studies of LLLT. (Pubmed.gov)

A laser is a device that generates light through a process of optical amplification based on the stimulated emission of electromagnetic radiation. There are four main classes of lasers as defined by the International Engineering Consortium (IEC standard 60825.) These classes indicate potential danger the radiation is to the eye.

a. Class 1/1M – CD player
b. Class 2/2M – laser pointer
c. Class 3R/3B – LLLT and CD and DVD writers
d. Class 4 – Surgical laser

LLLT is the application of light (usually a low powered laser or LED typically power range of (10mW–500mW). Light with a wavelength in the red to near infrared region of the spectrum (660nm–905nm), is generally employed because these wavelengths have the ability to penetrate skin, and soft/hard tissues (Figure 2) and are proven in clinical trials to have a good effect on pain, inflammation and tissue repair. The power density (irradiance) is usually between 5W/cm^2 and is applied to an injury or to a painful site for 30–60 seconds a few times a week for several weeks. The result is a reduction of inflammation, pain relief and accelerated tissue regeneration. In most cases the lasers/LEDs used for LLLT emit a divergent beam (not focused or collimated) because collimation is lost in tissue, but as a consequence ocular risks are also diminished over distance.

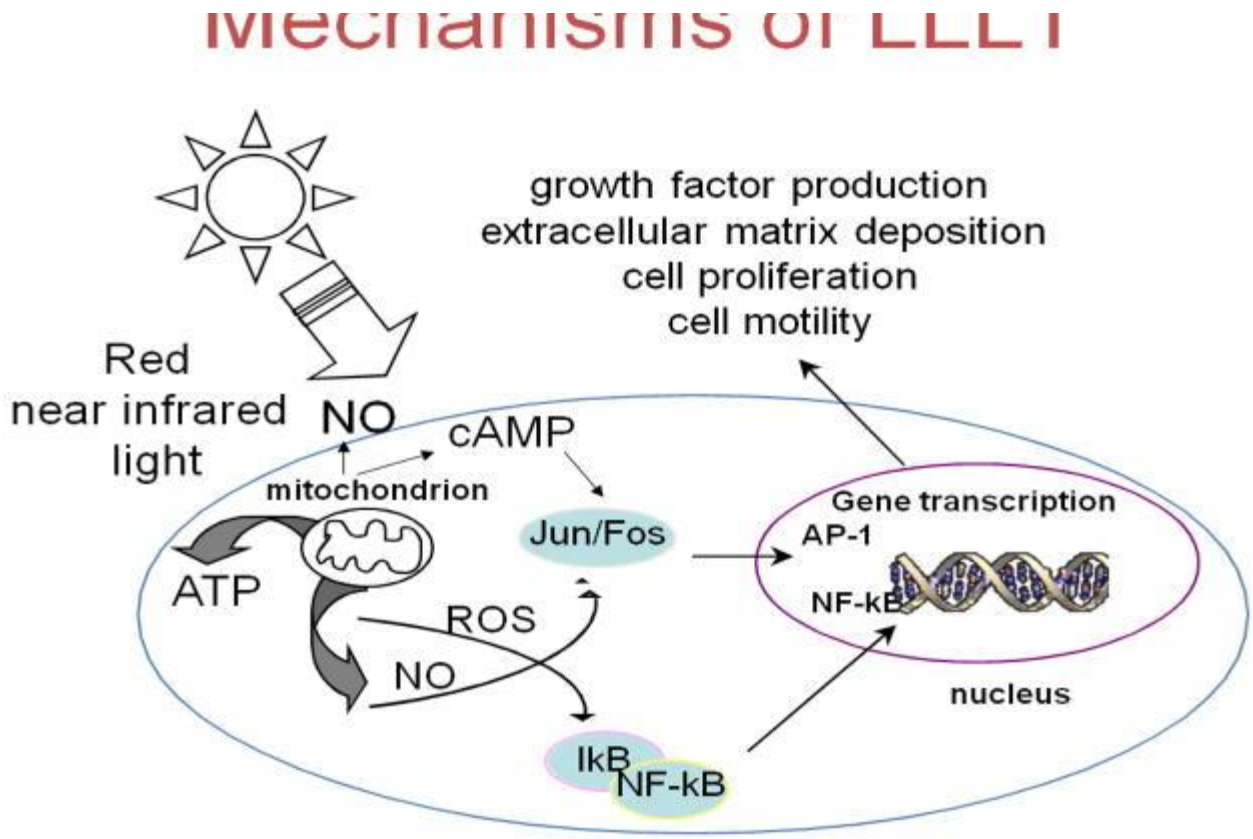
Figure 2.



Tissue optical window.

Mechanisms of LLLT (Figure 3)

Figure 3.



Mechanisms of LLLT.

For low-power visible or near-infrared light to have an effect on a biologic system, the photon must be absorbed by electronic absorption bands belonging to a photon acceptor or chromophore (first law of photobiology) [16]. A chromophore is a molecule (or portion of a molecule) which imparts a color to a compound (e.g. chlorophyll, hemoglobin, myoglobin, cytochrome c oxidase, other cytochromes, flavin, flavoproteins or porphyrins) [17]. The “optical window” in a tissue describes a range of wavelengths where the penetration of light into tissue is maximized by employing red and near-infrared wavelengths [18]. The optimum wavelength has been estimated to be around 810 nm. Mitochondria are “the cellular power plants” in our cells and as such they convert food molecules and oxygen into energy (ATP) by oxidative phosphorylation. It has been proposed that cytochrome c oxidase (COX) is the primary photo-acceptor for the red-NIR wavelength range in mammalian cells [19]. Nitric oxide (NO) produced in mitochondria can inhibit respiration by binding to COX and displace oxygen especially in injured or hypoxic cells [20]. It is proposed that LLLT can photo-dissociate NO from COX and reverse the mitochondrial inhibition of respiration due to excessive NO binding [21]. The process of light mediated vasodilation was first described by RF Furchgott [22] in 1968, and his research on the biological properties of nitric oxide eventually led to the award of a Nobel Prize in 1998 [23]. LLLT is able to produce a shift in the overall cell redox potential in the direction of greater oxidation by increasing reactive oxygen species (ROS) and decreasing reactive nitrogen species (RNS) [24–30]. The long-term effects of LLLT are thought to be due to the activation of various transcription factors by

the immediate chemical signaling molecules produce from mitochondrial stimulation by LLLT. The most important of these signaling molecules are thought to be ATP, cyclic-AMP, NO and ROS [16].

LLLT at low doses has been shown to enhance cell proliferation of fibroblasts [31–34], keratinocytes [35], endothelial cells [36] and lymphocytes [37,38]. The mechanism of proliferation is thought to result from photo-stimulation of the mitochondria leading to activation of signaling pathways and up regulation of transcription factors eventually giving rise to increases in growth factors [31,39–42]. LLLT can enhance neovascularization, promote angiogenesis and increase collagen synthesis to aid in the healing of acute [43] and chronic wounds [44–46]. It has been observed in many studies, that LLLT exhibits a biphasic dose response curve [47,48], where by lower doses of light are more effective than much higher doses. These low doses of light have demonstrated the ability to heal skin, nerves, tendons, cartilage and bones. This biphasic dose response curve may have important implications for LLLT for pain relief for the following reasons. Low-intensity LLLT stimulates mitochondria and raises mitochondrial membrane potential [49–51] and might be supposed to be more likely to increase metabolism and transport of action potentials in neurons rather than decrease it. However, much higher intensity LLLT produced by a focused laser spot acting on a nerve has the opposite effect, inhibiting mitochondrial metabolism in c-fibers and a-delta fibers and reducing mitochondrial membrane potential, thereby inducing a nerve blockade (see below).

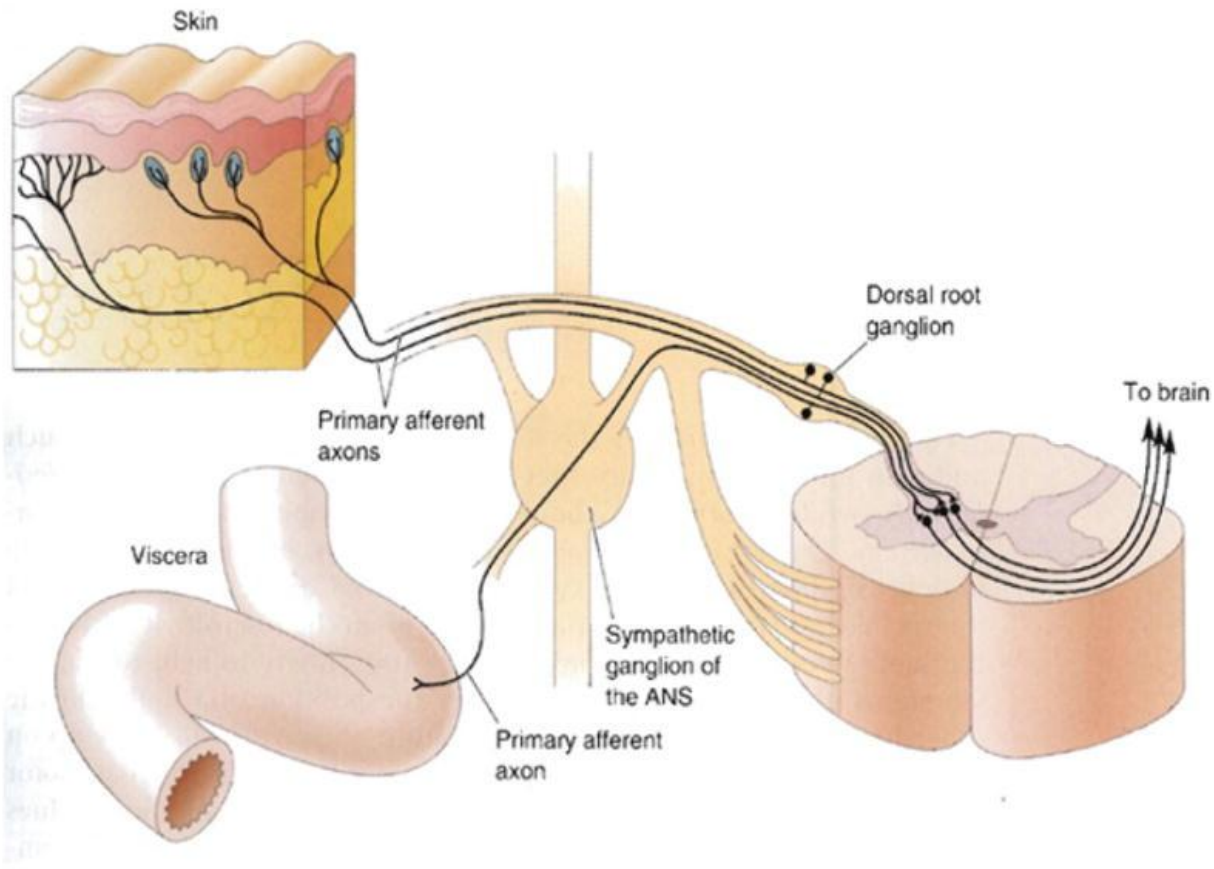
LLLT in the treatment of pain

Acute orthopedic conditions such as sprains [52,53], strains, post-surgical pain, a whiplash injury [54], muscular back pain, cervical or lumbar radiculopathy [55,56], tendinitis [57,58] and chronic conditions such as osteoarthritis [59–64], rheumatoid arthritis, frozen shoulder [65], neck and back pain [56], epicondylitis [66], carpal tunnel syndrome [67,68], tendinopathy [69], fibromyalgia [70], plantar fasciitis [70], post tibial fracture surgery [9] and chronic regional pain syndrome are amenable to LLLT. Dental conditions producing pain such as orthodontic procedures [71], dentine hypersensitivity [72], and third molar surgery [73] respond well to treatment with LLLT. Neuropathic pain conditions can also be treated such as post herpetic neuralgia [74], trigeminal neuralgia (10), and diabetic neuropathy [75]. Due to the wide spectrum of conditions one would surmise that multiple mechanisms can operate to achieve pain relief.

The peripheral nerve endings of nociceptors, consisting of the thinly myelinated A∂ and unmyelinated, slow-conducting C fibers, lie within the epidermis. This complex network transduces noxious stimuli into action potentials. Moreover these nerve endings are very superficial in nature and thus are easily within the penetration depths of the wavelengths used in LLLT (Figure 4). The cell bodies of neurons lie within the dorsal nerve root ganglion, but the elongated cytoplasm (axons) of the neurons extends from the cell body to the bare nerve endings in the surface of the skin. The direct effect of LLLT are initially at the level of the epidermal neural network, but the effects move to

nerves in subcutaneous tissues, sympathetic ganglia, and the neuromuscular junctions within muscles and nerve trunks.

Figure 4.



Afferent nerves.

LLLT applied with a sufficient level of intensity causes an inhibition of action potentials where there is an approximately 30% neural blockade within 10 to 20 minutes of application, and which is reversed within about 24 hours [76]. The laser application to a peripheral nerve does have a cascade effect whereby there is suppressed synaptic activity in second order neurons so that cortical areas of the pain matrix would not be activated.

Adenosine triphosphate (ATP) is the source of energy for all cells, and in neurons this ATP is synthesized by mitochondria while they are located in the dorsal root ganglion. These mitochondria are then transported along the cytoskeleton of the nerve by a monorail system of molecular motors. LLLT acts like an anesthetic agent, in that both LLLT and anesthetics have been shown to temporally disrupt the cytoskeleton for a matter of hours as evidenced by formation of reversible varicosities or beading along

the axons, which in turn cause mitochondria to “pile up” where the cytoskeleton is disrupted [77]. The exact mechanism for this effect is unknown but it is not a thermal action. It has been shown that LLLT at the correct dose decreases mitochondrial membrane potential (MMP) in DRG neurons and that ATP production is then reduced [78] so perhaps the lack of ATP could be cause of this neural blockade. The most immediate effect of nociceptor blockade is pain relief which occurs in a few minutes and has been shown by the timed onset of a conduction blockade in somatosensory-evoked potentials (SSEPs) [76]. This inhibition of peripheral sensitization not only lowers the activation threshold of nerves but also decreases the release of pro inflammatory neuropeptides (i.e. substance P and CGRP). In persistent pain disorders this reduction of tonic input to activated nociceptors and their synaptic connections, leads to a long-term down-regulation of second-order neurons [78]. The modulation of neurotransmitters is a further possible mechanism of pain relief, as serotonin and endorphin levels have been shown to increase in animal models [79,80] and following laser treatment of myofascial pain in patients [81]. Thus LLLT can have short, medium and long term effects. Fast acting pain relief occurs within minutes of application, which is a result of a neural blockade of the peripheral and sympathetic nerves and the release of neuromuscular contractions leading to in a reduction of muscle spasms [82,83].

In the medium term there is a decrease of local edema and a reduction of inflammation within hours to days [84]. The action of LLLT in reducing swelling and inflammation has been well established in animal models as well as in clinical trials. The numbers of inflammatory cells has been shown to be reduced in joints injected with protease [85], in collagen-induced rheumatoid arthritis [86], and in acute pulmonary inflammation [87]. The expression levels of pro-inflammatory cytokines have been shown to be reduced by LLLT in burn wounds [88], in muscle cryo lesions [89] and in delayed type hypersensitivity [90]. The long term effects of LLLT occur within a week or two and can last for months and sometimes years as a result of improved tissue healing.

LLLT parameters

For LLLT to be effective, the irradiation parameters (wavelength, power, power density, pulse parameters, energy density, total energy and time) need to be within certain ranges. The best penetrating wavelengths in the range of 760–850nm and may achieve a light density of 5mW/cm^2 at 5cm deep when the beam power is 1Watt and surface density is 5W/cm^2. There are four clinical targets for LLLT:

a. The site of injury to promote healing, remodeling and reduce inflammation.
b. Lymph nodes to reduce edema and inflammation.
c. Nerves to induce analgesia.
d. Trigger points to reduce tenderness and relax contracted muscle fibers.

Treatment times per point are in the range of 30 seconds to 1 minute. As little as one point may be treated in simple cases, but as many as 10 to 15 points may be treated for more complex dysfunction such as cervical or lumbar radiculopathy.

The potential hazards are mostly ocular, as some LLLT devices are lasers, though increasingly LLLT devices have become LEDs. In most cases, LLLT devices emit divergent beams (not focused or collimated), so the ocular risk diminishes over distance. Manufacturers are obliged to provide the nominal ocular hazard distance (NOHD) within their user instructions. ANSI 2 136.3 (2011) is the current definitive USA document on laser safety in healthcare environments (www.ansi.org) and IEC60825 is the International Standard. Part 8 provides guidelines for the safe use of laser beams on humans (www.iec.ch).

The North American Association for Laser Therapy conference in 2010 held a consensus meeting on safety and contraindications. Their main recommendations were:

I. Eyes - Do not aim laser beams into the eyes and everyone present should wear appropriate safety spectacles.
II. Cancer - Do not treat over the site of any known primary carcinoma or secondary metastasis unless the patient is undergoing chemotherapy when LLLT can be used to reduce side effects such as mucositis. LLLT however can be considered in terminally- ill cancer patients for palliative relief.
III. Pregnancy- Do not treat directly over the developing fetus.
IV. Epileptics - Be aware that low frequency pulsed visible light (<30Hz) might trigger a seizure in photosensitive, epileptic patients.

The adverse effects of LLLT have been reported to be no different from those reported by patients exposed to placebo devices in trials.

Orthopedic outcomes

According to the more than 4000 studies on pub.med.gov, it can be concluded that the majority of laboratory and clinical studies have demonstrated that LLLT has a positive effect on acute and chronic musculoskeletal pain. Due to the heterogeneity of populations, interventions and comparison groups, this diversity means that every single study has not been positive. Pain is a very complex condition which presents in different forms with an interplay of mechanical, biochemical, psychological and socioeconomic factors. It is extremely challenging to compare LLLT to other treatments, and LLLT regimens are complicated by different lengths of treatment, all without standardization of wavelengths and dosages. Currently, there have been no long-term (greater than 2 year follow up) human clinical studies that have evaluated LLLT. The overall positive short term clinical studies in addition to strong laboratory studies should give the clinical confidence that LLLT may be beneficial for many individuals suffering from musculoskeletal pain, regardless of the cause. Consideration of evidence based treatment studies for LLLT has led to the determination that LLLT is classified as experimental/investigational by insurance companies (BCBSKS 2013), while the American Academy of Orthopedic Surgeons has no recommendations for or against its use. With FDA approval for temporary relief of muscle and joint pain, this underlines the need for further well-designed clinical studies.

Conclusion

One has to be realistic about the therapeutic use of LLLT. The previous discussion has shown that LLLT is beneficial for pain relief and can accelerate the body's ability to heal itself. LLLT has a long history and strong basic science evidence, which supports its use in pain management. It has few side effects and is well tolerated by the elderly. A laser or LED does not correct situations involving structural deficits or instabilities whether in bone or in soft tissue. Also, LLLT should only be used as an adjuvant therapy for pain relief in patients with neuropathic pain and neurologic deficits. Successful outcomes, like all medical management, depend on good clinical skills linked with an understanding of the nature of injury, inflammation, repair, pain, and the mechanism of laser and LED effects.

Acknowledgments

Michael R Hamblin was supported by US NIH grant R01AI050875.

Proposed Mechanisms of Photo biomodulation or Low-Level Light Therapy

Lucas Freitas de Freitas [1,2], Michael R Hamblin [2,3,4,*]
- Author information
- Copyright and License information
PMCID: PMC5215870 NIHMSID: NIHMS797827 PMID: 28070154
The publisher's version of this article is available at IEEE J Sel Top Quantum Electron

Abstract

Photobiomodulation (PBM) also known as low-level laser (or light) therapy (LLLT), has been known for almost 50 years but still has not gained widespread acceptance, largely due to uncertainty about the molecular, cellular, and tissular mechanisms of action. However, in recent years, much knowledge has been gained in this area, which will be summarized in this review. One of the most important chromophores is cytochrome c oxidase (unit IV in the mitochondrial respiratory chain), which contains both heme and copper centers and absorbs light into the near-infra-red region. The leading hypothesis is that the photons dissociate inhibitory nitric oxide from the enzyme, leading to an increase in electron transport, mitochondrial membrane potential and ATP production. Another hypothesis concerns light-sensitive ion channels that can be activated allowing calcium to enter the cell. After the initial photon absorption events, numerous signaling pathways are activated via reactive oxygen species, cyclic AMP, NO and Ca2+, leading to activation of transcription factors. These transcription factors can lead to increased expression of genes related to protein synthesis, cell migration and proliferation, anti-inflammatory signaling, anti-apoptotic proteins, antioxidant enzymes. Stem cells and progenitor cells appear to be particularly susceptible to LLLT.
Keywords: Low Level Light Therapy, Mechanism, Mitochondria, Cytochrome c oxidase, Photobiomodulation, Light sensitive ion channels

HISTORICAL INTRODUCTION

The first evidence of the action of low-level laser irradiation came from the experiments of Dr. Endre Mester, at the Semmelweis Medical University (Hungary) in 1967. The experiment consisted of shaving the back of mice and implanting a tumor via an incision in the skin. Mester applied light from a ruby laser (694 nm) in an attempt to repeat one of the experiments described by McGuff in Boston [1]. McGuff had used the newly discovered ruby laser to cure malignant tumors both in rats and also tested it in human patients. Unfortunately (or perhaps fortunately for scientific discovery), Mester's laser had only a small fraction of the power possessed by McGuff's laser. Therefore Mester failed to cure any tumors, but did observe a faster rate of hair growth in the treated mice compared to the controls [2], calling this effect "laser biostimulation". He later used a HeNe laser (632.8 nm) to stimulate wound healing in animals, as well as in clinical studies [3]. For several decades, the profession believed that coherent laser light was necessary, but as of today, non-coherent light sources such as light emitting diodes

(LED) have proved to be just as efficient as lasers in promoting photobiomodulation (PBM) [4].

Low-level light therapy (LLLT) or PBM consists of the application of light with the purpose of promoting tissue repair, decreasing inflammation, and producing analgesia, usually using a low-power light source (laser or LED) [5]. Because of the low power, (usually below 500 mW depending on the target tissue) the treatment causes no evident temperature rise in the treated tissue and, therefore, no significant change in the gross tissue structure [6]. PBM/LLLT differs from other light-based treatments because it does not ablate and is not based on heating. It also differs from photodynamic therapy (PDT), which is based on the effect of light to excite exogenously delivered chromophores to produce toxic reactive oxygen species (ROS) [7].

With the advantage of being non-invasive, the applications of PBM are broad, going from pain relief to promoting the recovery of tendinopathies, nerve injuries, osteoarthritis and wound healing. The complete mechanism of action is still elusive, but the knowledge that has been gained so far is the subject of the present review. The importance of parameters in PBM will be discussed, together with the possible chromophores or photoacceptors, signaling molecules produced after photon absorption, transcription factors that may be activated to account for the lasting effects of a brief light exposure, downstream effector molecules that follow on, and specific mechanisms that may be applicable to the different cells and tissues being treated with PBM.

PARAMETERS OF PBM

The light parameters and the doses applied are fundamental in PBM. The most important parameters regarding the light source and the light doses are described on the following tables (table 1 and table 2, respectively):

Table 1.

Description of the irradiation parameters.

IRRADIATION PARAMETERS		
Irradiation Parameter	Measurement unit	Description
Wavelength	nm	Light is an electromagnetic form of energy with a wave-like behavior. Its wavelength is measured in nanometers (nm), and it is visible within the 400–700 nm range.
Irradiance	W cm^{-2}	It can also be called Power Density or Intensity, and corresponds to the power (in W) divided by the area (in cm^{-2}).

IRRADIATION PARAMETERS		
Pulse Structure	Peak Power (W) Pulse frequency (Hz) Pulse width (s) Duty cycle (%)	If the beam is pulsed, the Power should be called Average Power, which is calculated as follows: Average Power (W) = Peak Power (W) x pulse width (s) x pulse frequency (Hz)
Coherence	Coherence length depends on spectral bandwidth	Coherent light produces laser speckle, which is believed to play an important role on photobiomodulation interaction with cells and organelles.
Polarization	Linear polarized or circular polarized	Polarized light is known to lose its polarity in highly scattering media such as biological tissues, therefore this property is not considered very often on the effects of PBM.

Table 2.
Description of the light dose parameters.

LIGHT DOSE PARAMETERS		
Irradiation Parameter	Measurement unit	Description
Energy	Joules (J)	It cannot be mistook as dose, as it assumes reciprocity (the inverse relationship between power and time). It is calculated as: Energy (J) = Power (W) x Time (s)
Energy Density	J cm^{-2}	This is n important descriptor of dose, but it could be unreliable when we consider that it assumes a reciprocity relationship between irradiance and time.
Irradiation Time	s	Possibly the best way to prescribe and to record PBM would be to define the four parameters of table 1 and then define the irradiation time as the real "dose".
Treatment Interval	Hours, days or weeks	Different time intervals may result in different outcomes, but more data need to be gathered in order to define the extent of the differences between them.

Low level light therapy refers to the use of light in the red or near-infrared region, with wavelengths usually in the range of 600 to 700nm and 780 to 1100 nm, and the laser or LEDs typically having an irradiance or power density between 5 mW cm^{-2} to 5 W cm^{-2}.

This type of irradiation can be a continuous wave or a pulsed light consisting of a relatively low-density beam (0.04 to 50 J cm^{-2}), but the output power can vary widely from 1 mW up to 500 mW in order not to allow thermal effects [8]. The wavelength range between 700 and 780 nm has been found to be rather ineffective as it coincides with a trough in the absorption spectrum of cytochrome c oxidase (see later). Moreover red/NIR light is chosen because its penetration through tissue is maximal in this wavelength range, due to lower scattering and absorption by tissue chromophores. Although for many years it was thought that the monochromatic nature and coherence of laser light provided some sort of added benefit over non-coherent LED light, this view is no longer widely held. Continuous or pulsed light sources have both been used. The studies performed for PBM on acute pain and pre-operative analgesia show that a single treatment (usually only 30–60 seconds) is enough to cause analgesia, while for chronic pain and some degenerative conditions, more sessions are required [5].

It is known that if the incorrect parameters are applied, the treatment is likely to be ineffective. There is a biphasic dose response curve (or the phenomenon known as hormesis) in which when too low or too high doses (fluence (J/cm^2), irradiance (mW/cm^2), delivery time, or number of repetitions) can lead to no significant effect or, sometimes, excessive light delivery can lead to unwanted inhibitory effects [8], [9]. This biphasic response follows the "Arndt-Schulz Law" (which states that weak stimuli slightly accelerate vital activity, stronger stimuli raise it further until a peak is reached, whereas even stronger stimuli suppress it until a negative response is achieved), and has been demonstrated several times in low level light works [10]–[16].

For instance, Bolton irradiated macrophages with the same energy density (in J cm^{-2}) but with different irradiances (W cm^{-2}), and observed different results between the two conditions [17]; Karu and Kolyakov, in 2005, found that the stimulation of DNA synthesis rate is dependent on light intensity at a constant energy density of 0.1 J cm^{-2} with a clear maximum at 0.8 mW cm^{-2} [18]; Orion and co-workers worked with a constant energy density and different irradiances on an infarct model in rats after induced heart attack, and found that the beneficial effects were obtained at 5 mW cm^{-2}, while with irradiances as low as 2.5 mW cm^{-2} or as high as 25 mW cm^{-2} there were significantly less effects [11]; finally, Lanzafame and collaborators used a fixed energy density of 5 J cm^{-2} and variable irradiances, ranging from 0.7 to 40 mW cm^{-2}, observing that only with 8 mW cm^{-2} there were improvements on pressure ulcers in the treated mice [10].

There were some studies with constant irradiance and varying fluences. al-Watban and Andres, for instance, observed the effects of He-Ne laser on the proliferation of Chinese hamster ovary and human fibroblast. The light was delivered at a constant irradiance of 1.25 mW cm^{-2}, and a biphasic dose response was found with a peak at 0.18 J cm^{-2} [19]. Zhang and collaborators also found a biphasic dose response when they observed a maximum increase in human fibroblast cells after irradiation of light at 628 nm with fluence of 0.88 J cm^{-2}, while there was a marked reduction in the proliferation rate at 9 J cm^{-2} [20].

Regarding the time interval between treatments, Brondon and colleagues found that the best results for human HEP-2 and murine L-929 cells proliferation rates were achieved with two treatments per day, in comparison with one or four treatments per day. They used an LED with light at 670 nm and irradiance fixed at 10 mW cm^{-2}, and each treatment consisted on the delivery of 5 J cm^{-2} (the course was stopped after 50 J cm^{-2} had been delivered) [21].

There are also some systematic reviews and meta analyses of randomized, double-blind, placebo-controlled, clinical trials (RCTs) available in the literature. We can give as an example the review from Bjordal, who identified 14 RCTs of suitable methodological quality. 4 of them failed to report significant effects because the irradiance was either too low or too high, or because there was an insufficient delivery of energy [22]. Another review was performed by Tumilty with 25 RCTs of tendinopathies, 55% of which failed to produce positive outcomes because of an excessive irradiance delivery in comparison with the guidelines set by the World Association for Laser Therapy [23].

As we have seen, at low doses (up to 2 J cm^{-2}), PBM stimulates proliferation, whereas at higher doses (16 J cm^{-2} or higher) PBM is suppressive, pointing to the dose dependence of biological responses after light exposure [24]. Other authors, however, have observed stimulating effects outside the cited range [25], [26]. A number of different laser light sources, including helium-neon, ruby, and galliumaluminum-arsenide, have been used to deliver PBM in different treatments and on different schedules.

Many researchers fail to consider the importance of selecting the optimum parameters, or they do not have the necessary instrumentation or trained personnel to measure them accurately, resulting in treatment failures. Another cause of failure occurs whenever the terms are misused or wrongly reported. For instance, energy (J) or energy density (J cm^{-2}) are both usually referred to as "dose", but they are, in fact, different calculations, as demonstrated in table 2 [27].

MOLECULAR MECHANISMS OF PBM

Chromophores

Cytochrome c oxidase

Cytochrome C oxidase (Cox) is the terminal enzyme of the electron transport chain, mediating the electron transfer from cytochrome c to molecular oxygen. Several lines of evidence show that Cox acts as a photoacceptor and transducer of photosignals in the red and near-infrared regions of the light spectrum [28]. It seems that PBM increases the availability of electrons for the reduction of molecular oxygen in the catalytic center of Cox, increasing the mitochondrial membrane potential (MMP) and the levels of ATP, cAMP and ROS as well [29].

PBM increases the activity of complexes I, II, III, IV and succinate dehydrogenase in the electron transfer chain. Cox is known as complex IV and, as mentioned before, appears to be the primary photoacceptor. This assumption is supported by the increased oxygen consumption during low-level light irradiation (the majority of the oxygen consumption of a cell occurs at complex IV in the mitochondria), and by the fact that sodium azide, a Cox inhibitor, prevents the beneficial effect of PBM. Besides ATP and cAMP, nitric oxide (NO) level is increased, either by release from metal complexes in Cox (Cox has two heme and two copper centers) or by up-regulation of Cox activity as a nitrite reductase [30].

In fact, it was proposed that PBM might work through the photodissociation of NO from Cox, thereby reversing the mitochondrial inhibition of cellular respiration due to excessive NO binding [31]. NO is photodissociated from its binding sites on the heme iron and copper centers from Cox, where it competes with oxygen and reduces the necessary enzymatic activity. This allows an immediate influx of oxygen and, thus, the resumption of respiration and generation of reactive oxygen species. NO can also be photo-released from other intracellular sites, such as nitrosylated hemoglobin and myoglobin [32].

Retrograde mitochondrial signaling

One of the most accepted mechanisms for light-cell interaction was proposed by Karu[33], referring to the retrograde mitochondrial signaling that occurs with light activation in the visible and infrared range (Figure 1). According to Karu, the first step is the absorption of a photon with energy hv by the chromophore Cox. This interaction increases mitochondrial membrane potential (Δψm), causing an increase in the synthesis of ATP and changes in the concentrations of reactive oxygen species (ROS), Ca^{2+} and NO. Furthermore, there is a communication between mitochondria and the nucleus, driven by changes in the mitochondria ultrastructure, i.e. changes in the fission-fusion homeostasis in a dynamic mitochondrial network. The alteration in the mitochondrial ultrastructure induces changes in ATP synthesis, in the intracellular redox potential, in the pH and in cyclic adenosine monophosphate (cAMP) levels. Activator protein-1 (AP1) and NF-κB have their activities altered by changes in membrane permeability and ion flux at the cell membrane. Some complementary routes were also suggested by Karu, such as the direct up-regulation of some genes [34].

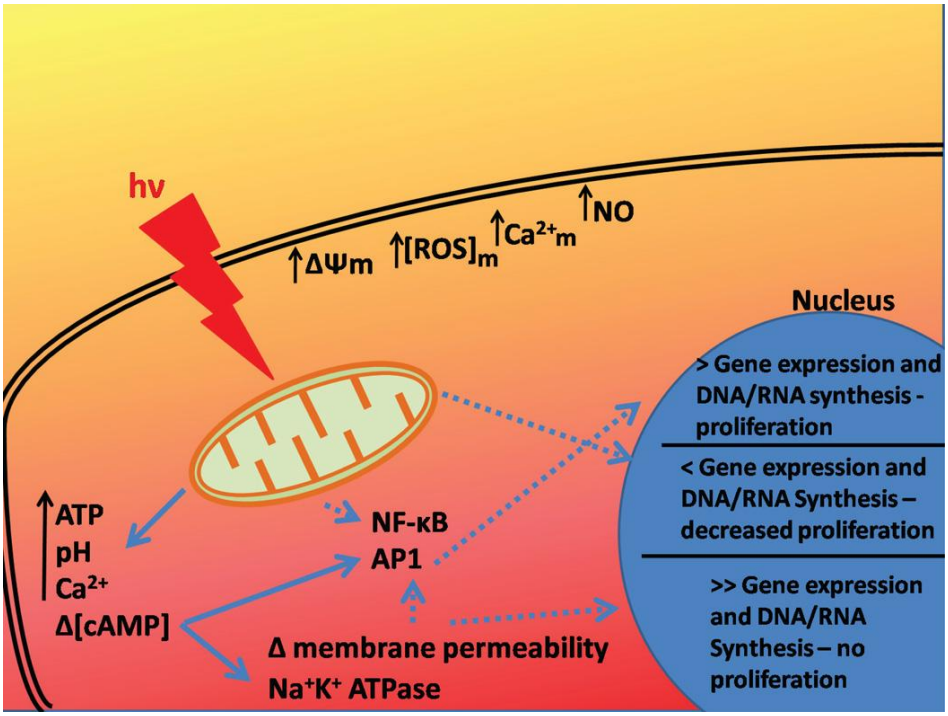


Figure 1.

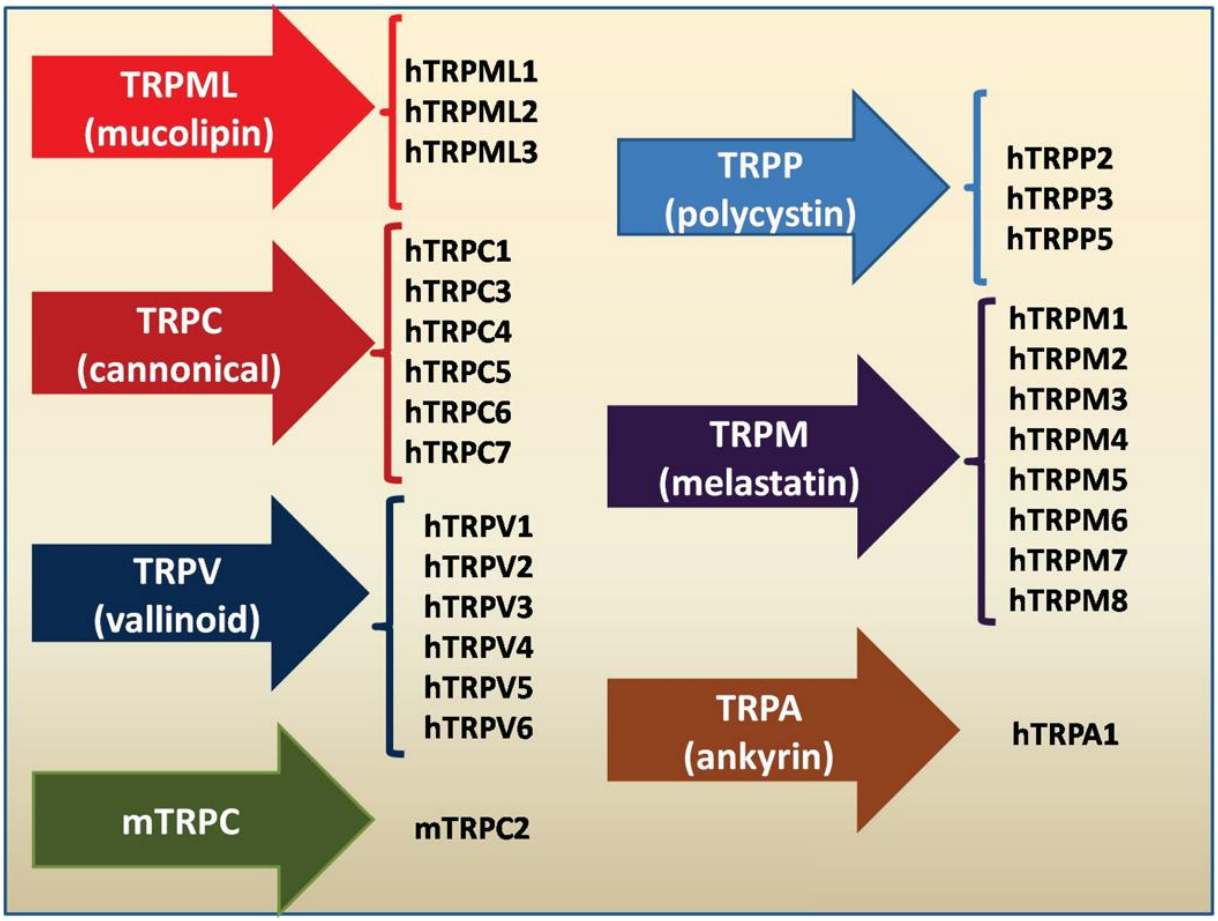
Scheme of mitochondrial retrograde signaling pathways as proposed by Karu. The main pathway is represented by continuous arrows, and the complementary ones are represented by segmented arrows.

Light sensitive ion channels

The most well-known ion channels that can be directly gated by light are the channelrhodopsins (ChRs), which are seven-transmembrane-domain proteins that can be naturally found in algae providing them with light perception. Once activated by light, these cation channels open and depolarize the membrane. They are currently being applied in neuroscientific research in the new discipline of optogenetics [35].

However, members of another broad group of ion-channels are now known to be light sensitive [36]. These channels are called "transient receptor potential" (TRP) channels as they were first discovered in a Drosophila mutant [36] and are responsible for vision in insects. There are now at least 50 different known TRP isoforms distributed amongst seven subfamilies [37], namely the TRPC (‘Canonical’) subfamily, the TRPV (‘Vanilloid’), the TRPM (‘Melastatin’), the TRPP (‘Polycystin’), the TRPML (‘Mucolipin’), the TRPA (‘Ankyrin’) and the TRPN (‘NOMPC’) subfamilies (see Figure 2). A wide range of stimuli modulate the activity of different TRP such as light, heat, cold, sound, noxious chemicals, mechanical forces, hormones, neurotransmitters, spices, and voltage. TRP are calcium channels modulated by phosphoinositides [38].

Figure 2.



All the seven subfamilies of Transient Receptor Potential Channels (TRP).

The evidence that light mediated activation of TRP is responsible for some of the mechanisms of action of PBM is somewhat sparse at present, but is slowly mounting. Mast cells are known to accumulate at the site of skin wounds, and there is some degree of evidence suggesting that these cells play a role in the biological effects of laser irradiation on promoting wound healing. Yang and co-workers demonstrated that after laser irradiation (532 nm), the intracellular [Ca^{2+}] was increased and, as a consequence, there was a release of histamine. If the TRPV4 inhibitor, ruthenium red, was used, the histamine release was blocked, indicating the central role of these channels in promoting histamine-dependent wound healing after laser irradiation [39].

It seems that TRPV1 ion channels are involved in the degranulation of mast cells and laser-induced mast cell activation. It was demonstrated that capsaicin, temperatures above 42°C and acidic pH could induce the expression of TRPV1 in oocytes, and these ion channels can be activated by green light (532 nm) in a power-dependent manner, although blue and red light were not able to activate them [40]. Infrared light (2,780 nm) attenuates TRVP1 activation by capsaicin in cultured neurons, decreasing the

generation of pain stimuli. TRPV4 is also attenuated by laser light, but the antinociceptive effect was less intense, therefore the antinociception in this model is mainly dependent on TRPV1 inhibition [41] The stimulation of neurons with pulsed infrared light (1,875 nm) is able to generate laser-evoked neuronal voltage variations and, in this case, TRPV4 channels were demonstrated to be the primary effectors of the chain reaction activated by the laser [42]. However, these effects after exposure to light above 1,500 nm might occur due to thermal effects, since water is the main absorber in this region of infrared spectrum. If it turns out that green light is primarily needed to activate ion channels then clinical applications may be limited due to lack of penetration into tissue.

Direct cell-free light-mediated effects on molecules

There have been some scattered reports that light can exert effects on some important molecules in cell free systems (in addition to the established effect on Cox). The latent form of transforming growth factor beta has been reported to be activated by light exposure [43]. Copper/Zinc Superoxide dismutase (Cu-Zn-SOD) from bovine erythrocytes that had been inactivated by exposure to pH 5.9 was reactivated by exposure to He-Ne laser light (632.7 nm) [44]. The same treatment also reactivated the heme-containing catalase. Amat et al. showed that irradiation of ATP in solution by 655 nm or 830 nm light appeared to produce changes in its enzyme reactivity, fluorescence and Mg2+ binding capacity [45]. However other workers were unable to repeat this somewhat surprising result [46].

Signaling Molecules

Adenosine triphosphate (ATP)

An increase in intracellular ATP is one of the most frequent and significant findings after PBM both *in vitro* and *in vivo* [47]. The stimulated synthesis of ATP is caused by an increased activity of Cox when activated by light. According to Ferraresi et al. [48], increased Cox activity is the mechanism of enhanced muscle performance when PBM is carried out before various types of exercises, for example. The authors found an increased ATP synthesis after LED (850±20 nm and 630±10 nm) therapy in different muscles (one with a predominantly aerobic metabolism, and other with mixed aerobic and glycolytic metabolism), just like previous data from Ferraresi et al. [49].

Extracellular ATP participates in a wide array of signaling pathways, known as purinergic signaling [50]. Originally discovered by Burnstock [51] as a non-adrenergic, non-cholinergic neurotransmitter, ATP purinergic signaling is mediated by P2Y G-protein-coupled receptors, and P2X ligand-gated ion channels [52]. ATP can be hydrolyzed to adenosine that carries out signals via the P1 G-protein-coupled receptor [53]. Up to the present date we are not aware of any studies that specifically show that extracellular (as opposed to intracellular) ATP or adenosine can be stimulated by PBM.

Cyclic AMP (cAMP)

Several workers have shown an increase in adenosine-3',5'-cyclic-monophosphate (cAMP) after PBM [54], [55]. Although it is tempting to suppose that this increase in cAMP is a direct consequence of the rise in ATP caused by light, firm evidence for this connection is lacking. It has been reported that cAMP-elevating agents, i.e. prostaglandin E_2, inhibit the synthesis of TNF and, therefore, down-regulate the inflammatory process. Lima and co-authors investigated the signaling pathways responsible for the anti-inflammatory action of PBM (660 nm, 4.5 J cm^{-2}) in lung and airways. They found reduced TNF levels in the treated tissue, probably because of an increase in cAMP levels. Furthermore, the authors demonstrated that the inflammation caused by LPS or by TNF in mice lungs was inhibited by cAMP-elevating agents. Rolipram, a cAMP-elevating agent, acts through inhibition of the enzyme phosphodiesterase, but it does not share this mechanism with low level light [54].

cAMP exerts its cellular effects via activation of three different kinds of sensors: cAMP-dependent protein kinase A (PKA) which phosphorylates and activates cAMP response element-binding protein (CREB), which then binds to CRE domain on DNA and in turn activates genes [56];cyclic nucleotide-gated channels (CNGC) [57] and exchange proteins directly activated by cAMP (Epac) [58].

Reactive oxygen species (ROS)

It was shown that PBM can produce mitochondrial ROS leading to activation of the transcription factor nuclear factor kappa B (NF-κB), which can act as a redox-sensor. The fact that the addition of antioxidants inhibits the activation of NF-κB by 810 nm light reinforces this assumption [59].

ROS are one of the classic "Janus face" mediators; beneficial in low concentrations and harmful at high concentrations; beneficial at brief exposures and harmful at chronic long-term exposures [60]. ROS are produced at a low level by normal mitochondrial metabolism [61]. The concept of mitohormesis was introduced to describe the beneficial of low controlled amounts of oxidative stress in the mitochondria [62]. However when the mitochondrial membrane potential is altered either upwards or downwards, the amount of ROS is increased. In normal cells, absorption of light by Cox leads to an increase in mitochondrial membrane potential and a short burst of ROS is produced. However when the mitochondrial membrane potential is low because of pre-existing oxidative stress [63], excitotoxicity [64], or inhibition of electron transport [63], light absorption leads to an increase in mitochondrial membrane potential towards normal levels and the production of ROS is lowered.

There are many different cellular systems that are designed by evolution to detect excessive levels of ROS and activate transcription factors to produce extra levels of

antioxidant defenses [65]. Hydrogen peroxide and lipid hydroperoxides [66] are thought to be the ROS most likely to carry out beneficial redox signaling by reversible oxidation of cysteine thiols in the sensor protein.

Calcium (Ca2+)

Changes in the mitochondrial ultrastructure may lead to alterations in Ca^{2+} concentration. The increment might be a result of Ca^{2+} influx from the extracellular environment and gated by the Ca^{2+} channel TRPV. There is evidence that cytosolic alkalinization can facilitate the opening of TRPV channels and, since laser irradiation can induce cellular alkalinization, PBM could induce TRPV opening and a consequent Ca^{2+} influx. In mast cells, this Ca^{2+} influx can mediate histamine release [67]. However it is also possible that light can directly activate TRPV channels as discussed above. It should be noted that PBM usually leads to an increase in intracellular Ca^{2+} as shown by fluorescent probes [68]. However when intracellular Ca^{2+} levels have been artificially raised (for instance by causing excitotoxicity with excess glutamate), then PBM can produce a drop in intracellular calcium and protect the neurons from dying [64]. The increase in calcium seen after PBM could also be a result of the release of Ca^{2+} from intracellular stores [69].

Calcium-sensitive signaling pathways are too numerous to cover in detail here, but include calcium sensitive enzymes like protein kinase C (PKC), calcium-calmodulin dependent kinase II (CamKII) and calcineurin (CaCN) [70], the extracellular calcium–sensing receptor (CaSR) [71], mitochondrial calcium signaling [72], calcium-sensitive adenylyl cyclase [73], and many others.

Nitric oxide (NO)

As mentioned above, NO is often found to be produced after PBM [74]. NO is a well-known vasodilator acting via stimulation of soluble guanylate cyclase to form cyclic-GMP (cGMP). cGMP activates protein kinase G, which causes reuptake of Ca^{2+} and opening of calcium-activated potassium channels. The fall in concentration of Ca^{2+} prevents myosin light-chain kinase (MLCK) from phosphorylating the myosin molecule, leading to relaxation of the smooth muscle cells in the lining of blood vessels and lymphatic vessels [75]. There are several other mechanisms by which NO could carry out signaling pathways, including activation of iron-regulatory factor in macrophages [76], modulation of proteins such as ribonucleotide reductase [77] and aconitase [78], stimulating ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase [79], and protein sulfhydryl group nitrosylation [80].

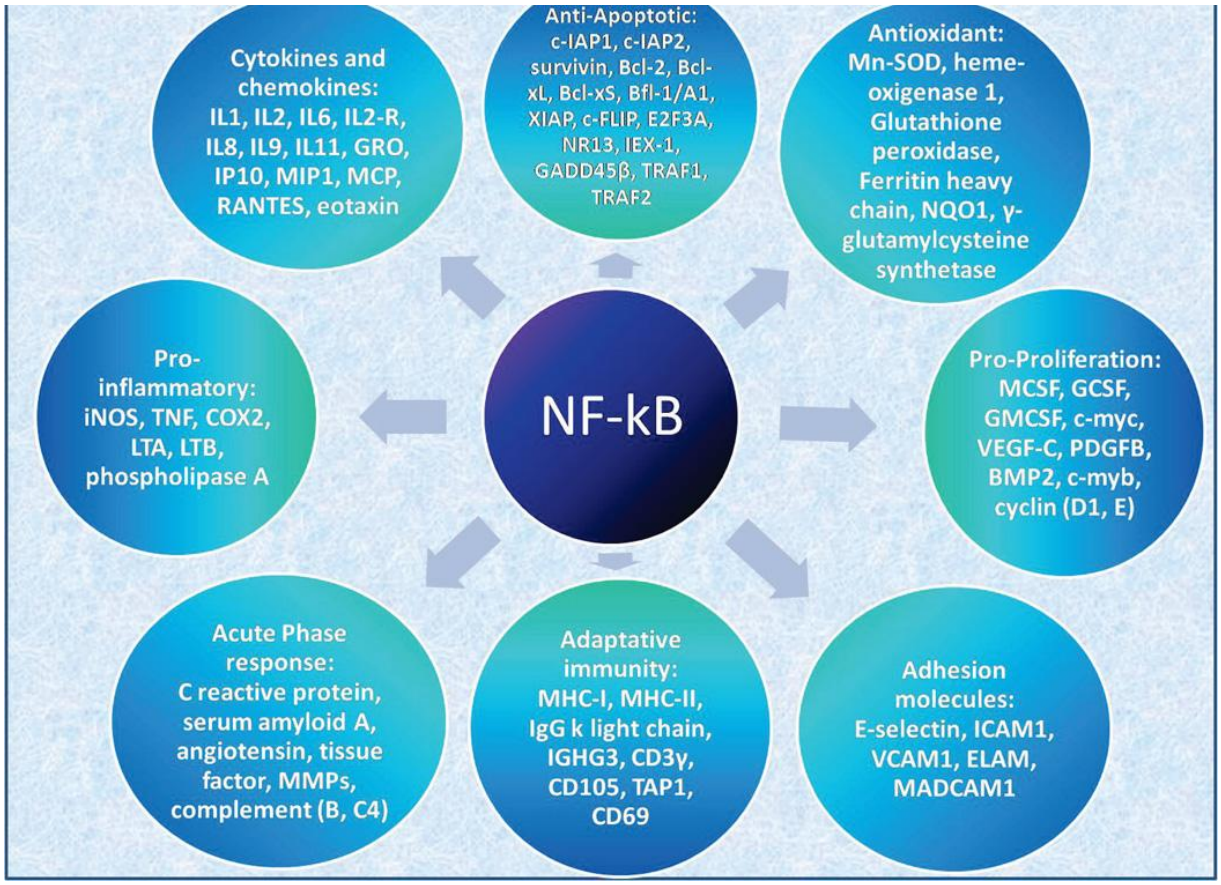
Activation of transcription factors

Nuclear factor kappa B (NFkB)

NF-κB is a transcription factor that regulates the expression of various genes related to many cellular functions, i.e. inflammatory and stress-induced responses and survival. Its activity is regulated by a negative feedback mediated by an inhibitor called IκB, which binds to NF-κB to inactivate it, or can undergo ubiquitination and go to proteasomal degradation in order to release NF-κB. The transcription factor, then, can be translocated to the nucleus and promote gene transcription. Several lines of evidence reveal that NF-κB is redox-sensitive, since ROS can directly activate it, or alternatively ROS could be involved in indirect activation of NF-κB via TNF, interleukin-1 (IL-1) and phorbol esters. PBM can boost ROS generation, and it was shown that light irradiation can induce NF-κB activation [59].

The increased NF-κB production after PBM stimuli leads to enhanced gene transcription that leads to reduced cell death, to cell proliferation, to cell migration [81] and enhanced neurological function. Figure 3 shows an overview of the different groups of genes that have NF-kB response elements.

Figure 3.



Overview of the different groups of genes and molecules that have NF-kB response elements. In principle these could be activated by NK-kB signaling pathway triggered by the ROS produced during LLLT

If the total energy density delivered is too high, however, the injury paradoxically tends to be exacerbated by increased oxidative stress, and an over-abundant activation of NF-κB. The biphasic dose effects of PBM are thought to occur due to an excessive generation of ROS, excessive production of NO, to the activation of some cytotoxic pathways, and to excessive NF-κB activation [82]. In addition, if the tissue is stressed or ischemic, mitochondria can synthesize NO that can displace oxygen from binding to Cox, but this leads to a reduced ATP synthesis and to an increased oxidative stress that can lead to inflammation when NF-κB is activated [83].

Classical mitochondrial inhibitors such as rotenone are known to decrease mitochondrial ATP levels, produce ROS and activate NF-κB. Low-level light still produces ROS and activates NF-κB, but in this case increases ATP levels. Antioxidants do not inhibit this ATP increase, suggesting that light augments the electron transport and potentially causes electron leakage (in the absence of antioxidants) and superoxide production [59].

RANKL

Receptor activator of nuclear factor kappa-B ligand (RANKL) is a transmembrane protein member of the TNF superfamily, involved in bone regeneration and remodeling (acting on osteoclast differentiation and activation). It is also a ligand for osteoprotegerin (OPG). The RANKL/OPG ratio determines whether bone is removed or formed during the remodeling process. The remodeling cycle consists in the increase in the expression of RANKL by osteoblasts, and subsequent binding to RANK receptor, which is highly expressed on osteoclastic membrane. This causes an expansion of the osteoclast progenitor pool, differentiation into mononucleated progenitor cells, increased survival, fusion into multinucleated osteoclasts and, finally, their activation. Osteoblasts can modulate this process by expressing OPG, which is a secretory soluble receptor and inhibitor of RANK receptor.

Parenti et al. investigated the RANKL/OPG ratio in osteoblast-like cells that were irradiated with GaAlAs laser (915 nm) using doses ranging from 1 to 50 J cm^{-2}. Although the differences were not statistically significant, there was a trend for a rapid and transitory increase in the RANKL/OPG ratio for all the tested doses. It seems that this ratio after PBM depends on the tissue and on the parameters used, since there is evidence of an increase in RANKL/OPG ratio in human alveolar bone-derived cells irradiated with 780 nm light, while in rat calvarial cells irradiated with 650 nm light the results were the opposite [84].

Hypoxia inducible factor (HIF-1α)

HIF-1α is a protein involved in cellular adaptation to hypoxia. It is stabilized at low oxygen tensions, but in the presence of higher oxygen concentrations it is rapidly degraded by prolyl hydroxylase enzymes, which are oxygen-dependent. HIF-1α activates genes that are important to the cellular response to hypoxic conditions, such as vascular endothelial growth factor (VEGF), VEGF-receptor, glucose carrier (GLUT-1) and phosphoglycerate kinase (PGK) genes. Since there is no significant changes in gross tissue oxygen concentration during PBM, HIF-1α activation may be mediated by the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway, by growth factors or cytokines [85]. Another possible explanation is that the sudden boost in cellular respiration caused by light activation of Cox depletes the low amount of oxygen that is present in hypoxic tissues but which is not being rapidly consumed because of inhibited electron transport. This sudden oxygen depletion then rapidly activates HIF-1α.

Cury demonstrated the pro-angiogenic effect of PBM using 660 nm and 780 nm light on skin flaps in rats. He observed that angiogenesis was induced by an increase in HIF-1α and VEGF expression, as well as by a decrease in matrix metalloproteinase 2 (MMP-2) activity [85]. Cury observed that only 660 nm light was able to increase HIF-1α expression, and although VEGF induction occurred in all light doses used, only 40 J cm^{-2} was able to induce angiogenesis, as well as an increase MMP-2 activity.

Akt/GSK3β/β-catenin pathway

Low-level light may exert a prosurvival effect on cells via the activation of AKT/GSK3β/β-catenin pathway. Basically, protein kinase B (also known as AKT) can be activated by LLL irradiation, and then interact with glycogen synthase kinase 3β (GSK3β), inhibiting its activity. GSK3B is a serine-threoninekinase which mediates various cellular signaling pathways, exerts metabolic control, influences embryogenesis, and is involved in cell death and in oncogenesis. There is evidence that this kinase is involved in the pathogenesis of Alzheimer's disease, since it promotes hyperphosphorylation of tau protein and causes the formation of neurofibrillary tangles (NTFs), both classic hallmarks of this disease.

The decreased activity of GSK3β is due to the fact that PBM-activated AKT increases the phosphorylation level of its Ser9 residue, which allows the N-terminus of GSK3β to bind with its own binding site. This leads to an accumulation of β-catenin and its translocation into the nucleus, where it can exert its prosurvival action. β-catenin is an important component of Wnt signalling pathway, responsible for the inhibition of axin-mediated β-catenin phosphorylation by GSK3β. This helps to stabilize the under-phosphorylated form of β-catenin, and ensure that it is no longer marked for proteasome degradation, so it can accumulate and travel to the nucleus. Once there, the prosurvival action of β-catenin relies on the increased TCF/LEF-dependent transcriptional activity. This prosurvival effect can be useful in the treatment of neurodegenerative diseases, such as Alzheimer's [86].

One of the most important regulators of apoptosis is Bax, a member of Bcl-2 family. It is translocated from the cytosol to the mitochondria when a pro-apoptotic stimulus is present, and this translocation is inhibited by PBM, according to Zhang et al. The authors hypothesized that GSK3β is the mediator between Akt and Bax during the PBM anti-apoptotic process. The authors found that GSK3β interacts with Bax and activates it, promoting its translocation directly, but PBM activates Akt which inhibits the activation of GSK3β, thus inhibiting Bax translocation. Using inhibitor compounds such as wortmannin and lithium chloride, there was a significant inhibition of the anti-apoptotic effect observed after PBM, suggesting that PI3K/Akt pathway (inhibited by wortmannin) and GSK3β translocation (inhibited by lithium chloride) play a key role in the protection against apoptosis caused by low level light. LiCl, however, was not able to reduce Bax translocation and apoptosis like PBM, so there must be other upstream regulators of Bax translocation during apoptosis. In conclusion, PBM exerted a pro-survival action through selectively activating the PI3K/Akt pathway and suppressing GSK3β/Bax pathway [87].

Akt/mTOR/CyclinD1 pathway

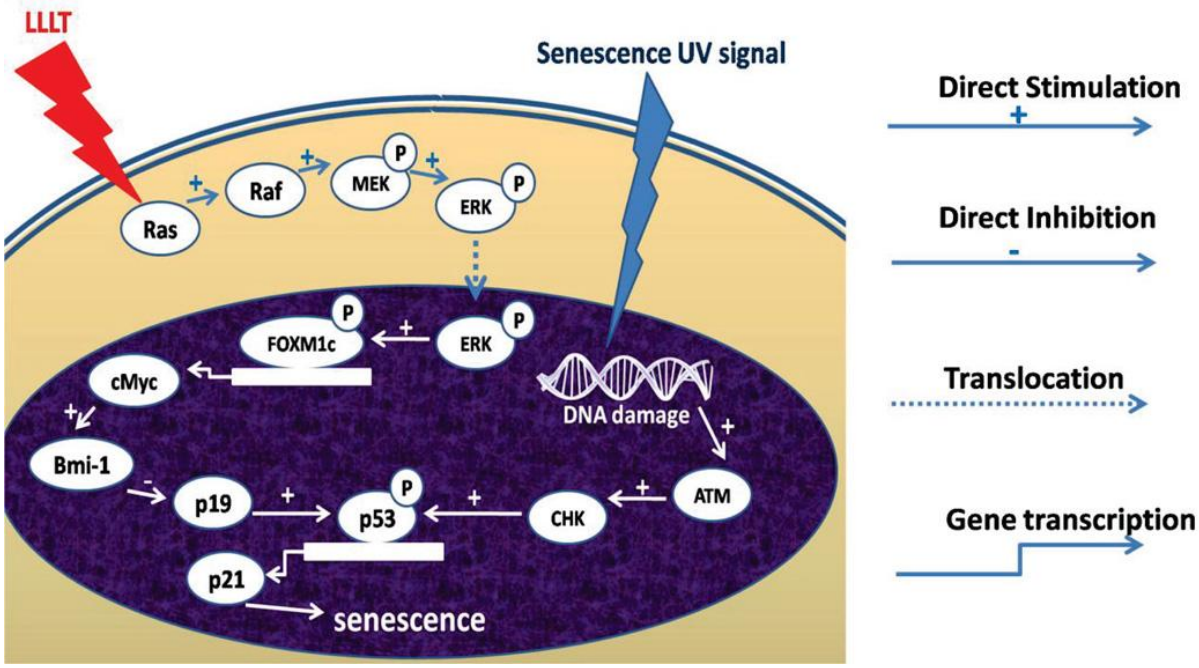
PBM has been demonstrated to be useful for stimulating proliferation of normal cells, but for dysplastic and malignant cells it could be dangerous. Sperandio et al. provided an example of this situation, observing that oral dysplastic cells, considered pre-malignant, had their viability increased after PBM (660 or 780 nm, 2 to 6 J cm^{-2}). Moreover, these workers showed higher expression of proteins related to cancer progression and invasion, i.e. Akt, HSP90, $pS6_{ser240/244}$, and Cyclin D1. The data suggest that Akt/mTOR/Cyclin D1 pathway was important for this phenotype differentiation, since the tested oral cancer cells showed higher levels of the signaling mediators that are part of this pathway [88].

ERK/FOXM1

Forkhead box protein M1 (FOXM1) is a protein involved in the regulation of the transition from G1 to S phase of the cell cycle and progression to mitotic division. Ling et al. investigated the protective effect of PBM using red light at 632.8 nm against senescence caused by UV light, and reported an activation of the ERK/FOXM1 pathway that caused a reduction in the expression of p21 protein and G1 phase arrest. Senescence was attenuated by over-expression of FOXM1c with or without PBM, and if FOXM1 was inhibited by shRNA, the effect of PBM in reducing cell senescence was abrogated. PBM promoted the nuclear translocation of extracellular signal-regulated kinase (ERK), increasing FOXM1 accumulation in the nucleus and the transactivation of c-Myc and p21 expression.

Inhibition of the mitogen-activated kinase (MEK)/ERK pathway with an MEK inhibitor PD98059 prevented the nuclear translocation of FOXM1 after PBM, suggesting that Raf/MEK/MAPK/ERK signaling is crucial for the anti-cell senescence effect of PBM mediated by FOXM1 [89]. Figure 4 summarizes these findings.

Figure 4.



A model of the signaling pathways for LLLT protecting cell from UVB-induced senescence.

PPARy

Peroxisome proliferator-activated receptors (PPAR) are mostly present in airway epithelial cells, but also in smooth muscle cells, myofibroblasts, endothelial cells of the pulmonary vasculature and in inflammatory cells such as alveolar macrophages, neutrophils, eosinophils, lymphocytes and mast cells. They are nuclear receptors with transcription factors that regulate gene expression. PPAR-y is involved in the generation of heat shock protein 70 (HSP-70), which is anti-inflammatory, while PPAR-c expression occurs due to an inflammatory response and are associated with massive lung injury and neutrophil infiltration in lungs of mice subjected to endotoxic shock [90]. Lima and co-authors reported a study in which rats were irradiated with 660-nm light (5.4 J) on the skin over the bronchus (chest). They observed a marked rise in the expression of PPAR mRNA after PBM, as well as increased PPAR-y activity in bronchoalveolar lavage (BALF) cells from animals subjected to laser treatment. In conclusion, Lima proposed that PBM can work as a homeostatic facilitator, increasing the expression of a transcription factor that is signaling the synthesis of HSP70 and other anti-inflammatory proteins[90].

RUNX2

Runt-related transcription factor 2 (RUNX-2) is related to osteoblastic differentiation and skeletal morphogenesis, acting as a scaffold for nucleic acids and regulatory factors that are involved in the expression of skeletal-related genes. It regulates the expression of

genes related to extracellular matrix components during bone cell proliferation. PBM can increase the expression of RUNX-2, contributing to a better tissue organization, even in diabetic animals as seen by Patrocínio-Silva [91].

Effector molecules

Transforming growth factor (TGF-β)

TGF-β is a strong stimulator of collagen production, inducing the expression of extracellular matrix components and inhibiting its degradation by inhibiting matrix metalloproteinases (MMPs). TGF-β expression is elevated during the initial phase of inflammation after an injury, and stimulates cellular migration, proliferation and interactions within the repair zone [92].

Dang and co-workers suggested that TGF-β/SMAD signaling pathway might play a role in PBM used for non ablative rejuvenation [93]. They found that 800 nm diode laser irradiation was able to induce collagen synthesis through the activation of TGF-β/SMAD pathway in a light dose-dependent manner. 40 J cm^{-2} was the most effective light dose in enhancing the gene expression of procollagen type I and IV, compared to 20 and 60 J cm^{-2}. The dermal thickness followed the results for the synthesis of collagen, demonstrating that this process was indeed dose-dependent [93].

Aliodoust et al. treated rats with 632.8 nm light and observed increased expression of TGF-β1 (one of the three isoforms of TGF-β) mRNA. TGF-β1 is responsible for the initial scar tissue formed at the wound site. It enhances tendon repair during the fibrosis period via the stimulation of cell proliferation and migration, as well as the synthesis of collagen and proteoglycans [92].

Oxidative stress

The inflammatory process involves an increase in ROS and RNS production, accompanied by a reduction in the activity of antioxidant defenses. This oxidative stress situation can activate NF-κB, as mentioned before, leading to modifications in the expression of genes for pro-inflammatory cytokines, growth factors, chemokines and adhesion molecules.

Assis et al. investigated the effects of PBM on muscle injury using 808 nm light (1.4 J), and observed reduced lipid peroxidation accompanied by a decreased COX-2 mRNA expression and an increased SOD mRNA expression after irradiation. There was a reduced formation of nitrotyrosine, indicating that iNOS activity was lower and, consequently, NO and peroxynitrite production was decreased. In conclusion, the inhibition of oxidative and nitrosative stress contributed to a decrease in the deleterious effects observed after muscle injury [94].

Pro- and anti-inflammatory cytokines

Many cytokines and inflammatory mediators have their levels altered by low-level light irradiation, regardless if they have pro- or anti-inflammatory actions, i.e. TNF, various interleukins, histamine, TGF-β, prostaglandins and eicosanoids. It seems that when inflammation is present, PBM exerts an anti-inflammatory action, but in the absence of inflammation, PBM provide pro-inflammatory mediators that could help in tissue remodeling and to mediate cell function. Wu and co-workers investigated the photoacceptor role of Cox and found that the excitation of Cox initiates a photoreaction that results in histamine release *in vitro*. The induced signals from mitochondria to cytosol cause alkalinization of the cytosol, which leads to the opening of TRPV channels. This results in an increment of [Ca^{2+}] and, consequently, in an enhanced histamine release [67]. Chen demonstrated in 2014 that an increased calcium influx occurred in mast cells after laser irradiation, and this caused histamine release that could help promoting wound healing. Furthermore, he found that during short-term muscle remodeling after cryoinjury, cytokines expression is also modulated by PBM, leading to a decreased expression of TNF and TGF-β[95].

Although NF-κB activation is known to be pro-inflammatory, PBM has a pronounced anti-inflammatory activity even with NF-κB activation. In fact, the anti-inflammatory effects of PBM could be abrogated if a NF-κB inhibitor is used. This probably occurs because the initial response to cell stress typical of NF-κB activation triggers another response to lower NF-κB activation after PBM had its therapeutic effect. Another possibility is that the initial pro-inflammatory response induced by PBM leads to the expression of eicosanoids that are able to decrease and to end inflammation [95].

Brain-derived neurotrophic factor (BDNF)

BDNF is part of the family of neurotrophins, molecules that exert actions on nerve cells. BDNF, specifically, seems to modulate dendritic structure and to potentiate synaptic transmission in the central nervous system. In order to investigate the effects of low-level light on BDNF levels, Meng et al. treated nerve cells with 632.8 nm light (doses from 0.5 to 4 J cm^{-2}). There was a regulatory role of PBM in neuroprotection and dendritic morphogenesis. PBM attenuated the decrease of BDNF, apparently by the ERK/CREB pathway, and this could be useful in the treatment of neurodegenerative disorders [96].

Vascular endothelial growth factor (VEGF)

Angiogenesis is a complex mechanism, requiring several cell types, mediators and signaling pathways. It is initiated by cell migration and invasion of endothelial cells, subsequent lumen formation and connection of the new vascular segments with preexisting ones, and finally, remodeling of extracellular matrix. This remodeling is

dependent on an adequate matrix metalloproteinases (MMPs) activity. VEGF and HIF-1α are critical to the angiogenic process.

PBM has been reported to induce angiogenesis in several experimental models. For example, Cury et al. observed a marked increase in the number of vessels in the skin flap of animals treated with 660 and 780 nm PBM, alongside with a marked increase in VEGF mRNA expression [85].

Hepatocyte growth factor (HGF)

HGF is a cytokine that regulates cell proliferation, motility, morphogenesis and exerts anti-apoptotic and anti-inflammatory activity during hepatic regeneration. The activation of its transmembrane tyrosine kinase receptor, called Met receptor, leads to autophosphorylation of tyrosine residues and phosphorylation of downstream signaling molecules, such as PI3K and MAPK pathway proteins. Araújo and co-workers observed that, after 632.8 nm PBM, hepatectomized animals showed an increase in the expression of HGF followed by increased phosphorylation of Met and its downstream signaling molecules Akt and ERK. This indicates that PBM could enhance liver regeneration after hepatectomy [97].

Basic fibroblast growth factor (bFGF) and keratinocyte growth factor (KGF)

Growth factors play a key role in the wound healing process, mediating the transfer of signals between the epithelium and the connective tissue, especially bFGF and KGF. bFGF is known to be a potent mitogen and chemoattractant for endothelial cells and fibroblasts, as well as accelerating the formation of granulation tissue and to induce re-epithelization. KGF is produced by fibroblasts and exerts a paracrine action on keratinocytes, therefore, it is responsible for the proliferation and migration of epithelial cells, as well as for the maintenance of the epithelium normal structure.

When gingival fibroblasts from a primary culture were irradiated twice with 660 or 780 nm low-level light in a study from Damante et al., production of KGF and bFGF was increased. Red light was more effective in stimulating KGF production, but no significant change in bFGF production was seen with red light. Near-infrared light, however, was capable of inducing bFGF release [98]. These results could explain how PBM can help the wound healing process.

Heat Shock Proteins (HSP)

Heat shock protein 27 (HSP27) is an important member of the small HSP family, with an ATP-independent chaperone activity that is produced in response to oxidative stress in order to modulate inflammation and to regulate the dynamics of the actin cytoskeleton. When HSP27 is activated, it facilitates the phosphorylation of IκB, causing it to be

degraded in the proteasome and increasing NF-κB activity. It also contributes to the regulation of NO and ROS production, iNOS expression and TNF secretion. However HSP27 plays a negative role in TNF-mediated IκB kinase (IKK) activation. The results of a study performed by Lim and co-workers with HSP27-silenced cells showed that 635 nm light irradiation was not able to decrease ROS generation if HSP27 was not present, indicating that this chaperone plays an important role in ROS decreasing during inflammation and PBM [99].

HSP70 is part of the normal wound healing process, alongside IL-6 and TGF-β1. Visible (532 nm) and NIR (815 nm) light have been demonstrated to induce HSP70 expression in treated skin cells, and this is important for skin rejuvenation interventions, since there is a consequent effect consisting on the assistance of the correct folding and transport of newly synthesized collagen [93].

HSP90 is another chaperone, which assists the maturation of Akt enabling it to perform its downstream actions. Increased activity of chaperones is certainly not desired in cancer, but it could be useful in healing processes. Sperandio et al. found higher levels of HSP90 in laser-treated cells, and an isoform of this chaperone, HSP90N, which has an oncogenic potential, was found in the experimental groups. This isoform is commonly overexpressed in tumor tissues and is secreted by advantage stages of melanoma [88].

Cellular mechanisms

Inflammation

Lim and co-workers found that 635 nm light irradiation at low power can lead to an anti-inflammatory effect by inhibiting prostaglandin E2 (PGE2) production and cyclooxygenase 1 and 2 (COX-1 and COX-2) mRNA expression. The light irradiation was able to decrease intracellular ROS, which mediate the expression of calcium-dependent phospholipase A2 (cPLA2), secretory phospholipase A2 (sPLA2), and COX-2, and also inhibit the release of PGE2 [99].

PGE2 synthesis is dependent on NF-κB modulation of the cellular signaling mechanism. NF-κB is found in the cytosol in its dimeric form of NF-κB/IκB (the latter is an inhibitory protein). Pro-inflammatory stimuli, such as LPS, are able to activate the NF-κB upstream signaling regulator IκB kinase (IKK), responsible for the phosphorylation and degradation of IκB. The free NF-κB is translocated to the nucleus and induces the expression of pro-inflammatory genes [8]. Lim demonstrated that 635 nm light irradiation suppressed the release of PGE2, possibly through a mechanism related to the inhibition of NF-κB pathway. It did not affect the phosphorylation of IκB, IKK and NF-κB in HSP27-silenced human gingival fibroblasts (hGFs), suggesting that NF-κB modulation by 635 nm light through HSP27 is required for the down-regulation of pro-inflammatory gene expression in these fibroblasts [99].

Macrophages are important antigen-presenting cells, and are involved in the induction of primary immunologic response. Interferon gamma (IFN-γ) polarization (either via classical or M1 activation) programs monocytes for increased phagocytic activity, as well as for anti-tumor activity and allergy suppression. Recently, Chen reported that 660 nm PBM could promote M1 polarization of monocytes, and influence the expression of cytokines and chemokines at the level of mRNA and protein expression. The effect was dose-dependent, since the optimal light dose found was that of 1 J cm^{-2}, compared to 2 and 3 J cm^{-2}. Furthermore, the author could also clarify the mechanisms of epigenetic regulation by PBM in immune cells. Modifications on histones, usually carried out by histone acetyl- or methyltransferases, could be induced by PBM: histone H3 and H4 acetylation and H3K4 trimethylation in the TNF gene promoter area, and histone H3 acetylation in the IP-10 gene promoter region. M1-related immunoregulation is important for antiviral immunity, antitumor immunity, and for the pathogenesis of infammation in autoimmune conditions, therefore PBM could help promoting anti-viral and anti-tumor immunity, but could enhance autoimmune and rheumatoid diseases [95].

Cytoprotection

Studies have shown that PBM in vitro protects cells at risk from dying due to treatment with various different toxins. Methanol, for instance, generates a toxic metabolite (formic acid) that inhibits cytochrome c oxidase. Since PBM enhances mitochondrial activity via stimulation of cytochrome c oxidase, it also promotes cell survival during formic acid toxicity. This was demonstrated by Eells, who used red light (670 nm) in a rodent model of methanol toxicity and found that the light irradiation induced a significant recovery of cone- and rod-mediated function in the retina of rats after methanol intoxication, as well as a protection against histological damage resulting from formic acid [100].

Cyanide is another toxic compound that can have its effects attenuated by PBM. Potassium cyanide-induced apoptosis of neurons was decreased with a pretreatment with 670 nm light. This is explained by the fact that PBM decreased the expression of caspase-3 (commonly increased by cyanide) and reversed the cyanide-induced increased expression of Bax, while decreasing the expression of Bcl-2 and inhibiting ROS generation [101]. Wong-Riley and co-workers show that LED pretreatment was not able to restore enzymatic activity in cells to control levels after cyanide toxicity, but it successfully reversed the toxic effect of tetrodotoxin, especially with 670 and 830 nm light. These wavelengths correspond to the peaks in the absorption spectrum of cytochrome c oxidase, suggesting that this photobiomodulation is dependent of the up-regulation of Cytochrome c oxidase [102].

PBM can be useful in the treatment of Alzheimer's disease, since low-power laser irradiation promotes Yes-associated protein (YAP) cytoplasmic translocation and amyloid-β-peptide (Aβ) inhibition. Aβ deposition is a known hallmark of Alzheimer's disease, while YAP translocation is involved in the regulation of Aβ-induced apoptosis. Zhang published a study demonstrating that 832.8 nm light irradiation is able to reduce

Aβ-induced toxicity by inhibiting apoptosis through the activation of Akt/YAP/p73 signaling pathway [103].

Proliferation

Several cell types can have their proliferation levels increased by PBM. Keratinocytes, for example, showed an enhanced proliferation after 660 nm light irradiation, accompanied by an increased expression of Cyclin D1 and a faster maturation of keratinocytes in migration to the wound sites, via the expression of proteins involved in the epithelial proliferation process, namely p63, CK10 and CK14. This is useful for the improvement of epithelial healing [104]. Furthermore, fibroblasts irradiated with 632.8 nm light had their proliferation stimulated and their cell viability increased, demonstrating the stimulatory effect of PBM and the usefulness of this therapy in the wound healing process [105].

Vascular endothelial cells exposed to 635 nm irradiation proliferate faster than non-irradiated cells, showing a decreased VEGF concentration. This suggests that laser-induced cell proliferation is related to a decrease in VEGF concentration. 830 nm irradiation decreased TGF-β secretion by the endothelial cells [106].

Amid et al. published a review about the influence of PBM on the proliferation of osteoblasts. According to the studies reviewed by the authors, wavelengths between 600 nm and 1000 nm have been used, and resulted in positive effects on dentistry, on anti-inflammatory process and on osteoblastic proliferation [107].

Fibroblasts irradiated with 632.8 nm light had their proliferation stimulated and their cell viability increased, demonstrating the stimulatory effect of PBM and the usefulness of this therapy in the wound healing process.

Migration

Tendon healing requires migration of tenocytes to the injured area, with consequent proliferation and synthesis of extracellular matrix. Tsai and co-workers evaluated the effect of 660 nm light on rat Achilles tendon-derived tenocytes, and found that dynamin-2 expression was enhanced and the migration was stimulated *in vitro*. Inhibiting dynamin-2 with dynasore suppressed this stimulatory effect of PBM, leading to the conclusion that tenocyte migration stimulated by low-level light was mediated by the up-regulation of dynamin-2 [108].

Other cell types are also influenced by light irradiation. Melanocytes, for instance, showed an enhanced viability and proliferation after blue and red light irradiation. Melanocytes migration was enhanced by UV, blue and red light in lower doses, but a non-stimulatory effect was observed for higher light doses. Blue light seemed to be more effective compared to UV and red lasers [109]. Human epidermal stem cell migration and proliferation were increased alongside an increased phosphorylation of autocrine extracellular signal-regulated kinase (ERK), which contributed to accelerated wound re-epithelialization [110]. Finally, 780 nm irradiation seemed to be able to accelerate fiber sprouting and neuronal cell migration, at least in embryonic rat brain cultures. Large-size neurons with a dense branched interconnected network of neuronal fibers were also observed after laser irradiation. These results can be seen in Rochkind's work, and may contribute for future treatment modalities for neuronal injuries or diseases [111].

Protein synthesis

As mentioned before, PBM was able to increase the expression of proteins related to the proliferation and maturation of epithelial cells: p63, CK10 and CK14 [104]. In fact, low level light can increase the expression of several other proteins. A good example is the enhanced collagen I expression by fibroblasts 2 days after 810 nm light irradiation, as demonstrated by Frozanfar and co-workers in 2013 [112]. Moreover, osteoblasts irradiated with 830 nm light increased the expression of proteins and proteoglycans such as osteoglycin and mimecan. Osteoglycin is a leucine-rich proteoglycan, once called osteoinductive factor, easily found in bone matrix, cartilage cells and connective tissues. They play a regulatory role in cell proliferation, differentiation and adhesion of osteoblastic cells, therefore PBM applied on the early proliferation stage of osteoblasts are important for the stimulation of bone formation, in concert with some growth factors and matrix proteins [113].

Stem cells

It appears that stem cells are particularly sensitive to light. PBM induces stem cell activity shown by increased cell migration, differentiation, proliferation and viability, as well as by activating protein expression [114]. Mesenchymal stem cells, usually derived from bone marrow, dental pulp, periodontal ligament and from adipose tissue, proliferate more after light irradiation (usually with wavelengths ranging from 600 to 700 nm). Since stem cells in their undifferentiated form show a lower rate of proliferation, this may be a limiting factor for the clinical effectiveness of stem cell therapies, PBM offers a viable alternative to promote the translation of stem cell research into the clinical arena [115].

Min and co-workers reported that the cell viability of adipose-derived stem cells was found to be increased after irradiation with 830 nm light. Their *in vivo* results also revealed elevated numbers of stem cells compared to the control group [116]. Epidermal stem cells can also be influenced by light, as demonstrated by Liao et al. The

authors reveal that 632.8 nm light has photobiological effects on cultured human epidermal stem cells, such as an increase in proliferation and migration *in vitro* [110]. Soares observed a similar effect on human periodontal ligament stem cells irradiated with a 660 nm diode laser [117].

Tissue mechanisms

Muscles

We already mentioned the positive results for PBM in muscle recovery, reported by Ferraresi et al. The authors demonstrated the usefulness of PBM in muscle recovery after injury. The authors concluded that it takes between 3 and 6 hours for the PBM to exert maximum effect on the muscle physiology, consisting of increased matrix metalloproteinase activity and ATP synthesis. This effect could still be observed 24 hours after the laser irradiation [49].

Rochkind and co-workers have also worked with PBM applied to muscles, investigating the influence of low power laser irradiation on creatine kinase (CK) and the amount of acetylcholine receptors (AChRs) present in intact gastrocnemius muscle *in vivo*, as well as the synthesis of DNA and of CK in muscle cells *in vitro*. The authors found that PBM significantly increased CK activity and AChR level in one and two months, when compared to control animals. The biochemical changes on muscle cells might be due to a trophic signal for increased activity of CK, which leads to a preservation of a reservoir of high-energy phosphate that is available for rapid ATP synthesis [118].

Brain

Regarding the neurological field, PBM can lead to cognitive benefits and memory enhancement in case of brain damage caused by controlled cortical impact (CCI). Khuman and co-workers found that a 500 mW cm^{-2} laser irradiation (60 J cm^{-2}) for two minutes improved spatial learning and memory of mice with CCI, and this was not observed in sham-injured mice. The authors observed a brief increase in the temperature of brain, but it returned to baseline before 5 minutes of irradiation. They also observed reduction of microgliosis at 48 h. Low level light can be useful in traumatic brain injury (TBI) treatment, since suboptimal light doses demonstrated to affect spatial memory, as assessed by visible platform trials, even in the absence of non-spatial procedural learning, which is hippocampus-independent [82].

Near-infrared (NIR) light exerts a protective effect on neurons, but the mechanisms are not fully understood. However, two mechanisms may be involved, and the first that will be discussed is the direct action of NIR light on the cells, improving mitochondrial function, reducing inflammation, and helping the brain to repair itself. Xuan et al

reported that transcranial NIR light could stimulate the process of neurogenesis in the hippocampus and subventricular zone (SVZ) in mice with CCI TBI [119]. These newly formed neuroprogenitor cells could travel to the injured region of the cortex to help in the repair of the damaged region. In another study the same group showed that BDNF was increased in the hippocampus and SVZ at one week post TBI, and that at 4 weeks post TBI there as an increase in synaptogenesis in the cortex showing that new connections between existing brain cells could be stimulated by light [120].

The second mechanism is based on the hypothesis that NIR can trigger a systemic response, this time not so directly, suggesting the involvement of one or more circulating molecules or cell types. This assumption is based on studies reporting remote effects on tissues after irradiation of NIR light on specific sites, such as skin wounds. Another study reported brain protection in mice after remote irradiation with NIR light to the dorsum of the animals, without any direct irradiation on the head. One possibility to explain these remote effects is the stimulation of mast cells and macrophages, which could help to protect cells in the brain, as well as the modulation of inflammatory mediators, like the down-regulation of pro-inflammatory cytokines and up-regulation of anti-inflammatory cytokines. Another possibility is the involvement of bone-marrow derived stem cells, since NIR light can increase the proliferation of c-kit-positive cells located in the bone marrow of the skull, which are then recruited to damaged tissues, especially the myocardial infarct site. These progenitor cells can, alongside with immune cells, secrete trophic and pro-survival factors such as nerve growth factor (NGF) and VEGF. Finally, mitochondria itself could be secreting an unidentified extracellular signal, called by Durieux et al. a “mitokine”, which is then transmitted to remotely located cells[82].

Nerves (repair and pain)

Some clinical studies have demonstrated the efficacy of laser-induced analgesia [121], [122], usually with a low power red or near-infrared laser, and it seems that the pain reduction is due to a conduction block of central and peripheral nerve fibers and to the release of endorphins. In this field, for instance, Chan and co-workers used a Nd:YAG pulsed laser (1064 nm) with average power 1.2 W and power density 0.3–0.45 J cm^{-2} in a randomized, double-blind clinical trial, and demonstrated the efficacy of this treatment on pulpal analgesia of premolar teeth[123].

Analgesia mediated by low level light therapy is due to various effects, such as light absorption by mitochondrial chromophores (mainly Cox) biomodulation, vasodilation, stimulation of cell division, release of NO, increase in cortisol levels and protein synthesis, increase in intracellular calcium concentration and increased activity of the antioxidant enzyme superoxide dismutase. Serra and Ashmawi investigated recently if serotonin played a role in PBM-induced analgesia, but their results indicated that this effect is mediated by peripheral opioid receptors, but not by peripheral serotoninergic receptors [124].

Low-level light therapy can be used for inhibition of pain and for pathological conditions associated with the nervous system. In 2011, Yan et al. postulated that PBM could suppress afferent fiber signaling as well as modulate synaptic transmission to dorsal horn neurons, including inhibition of substance P, and this can lead to long-term pain depression [125]. PBM exerts potent anti-inflammatory effects in the peripheral nervous system, can reduce myocardial infarction, promotes functional recovery and regeneration of peripheral nerves after injury, and can improve neurological deficits after stroke and TBI [82].

Light with irradiance higher than 300 mW cm^{-2}, when absorbed by nociceptors, can inhibit Aδ and C pain fibers, slowing of conduction velocity, reducing of the compound action potential amplitude, and suppression of neurogenicin inflammation. In case of PBM, the light can block anterograde transport of ATP-rich mitochondria in dorsal root ganglion neurons. This inhibition is completely reversible within 48 hours, and leads to the formation of varicosities, which are usually associated with the disruption of microtubules (interruption of fast axonal flow can reduce ATP availability, which is necessary for the polymerization of microtubules and for the maintenance of the resting potential) [83].

Healing (bones, tendons, wounds)

Regarding bones, low power laser irradiation is not believed to affect osteosynthesis, but it is likely that it creates environmental conditions that accelerate bone healing. PBM stimulates proliferation and differentiation of osteoblasts *in vivo* and *in vitro*, leading to an increased bone formation, accompanied by an increase in the activity of alkaline phosphatase (ALP) and in osteocalcin expression. This indicates that laser irradiation can directly stimulate bone formation and, according to Fujimoto et al., this effect can be attributed to an increased expression of insulin-like growth factor (IGF), although other differentiation factors might be involved as well, such as BMPs. BMPs-2, −4, −6 and −7 are members of the TGF-β superfamily, and potent promoters of osteoblastic differentiation and of bone formation (promoting the change of mesenchymal cells into chondroblasts and osteoblasts) [126].

According to Fujimoto, BMP-2 might be most involved in the effects of PBM on bone. PBM stimulated mineralization *in vitro* via increased gene and protein expression of BMPs and Runx-2, as well as differentiation of osteoblasts into MC3T3-E1cells. Since BMPs are one of the most important and potent bone-inductive mediators and are expressed in skeletal tissues, it is possible that the bone nodules formed after PBM are mediated in part by BMP-2 expression [126].

The balance between oxidants and antioxidants is directly related to the time and quality of the wound healing process[127]. This process can be divided in four overlapping phases: hemostasis, inflammation, proliferation and remodeling or resolution. Hemostasis is initiated as soon as the blood vessels are damaged, and consists on the

adherence of platelets to the extracellular matrix and further releasing of growth factors (mostly platelet-derived growth factor, PDGF and TGF-β), culminating in the production of thrombin which acts on fibrinogen to produce a fibrin clot. Thrombin also acts as a chemotactic agent and proliferating agent on monocytes, keratinocytes, fibroblasts and endothelial cells, therefore a defective thrombin activity can lead to a delay in the wound healing process. Hoffman reported that PBM could be beneficial in promoting healing when there is a defect in the hemostasis process [128].

Hair

Different mechanisms have been proposed to explain the reason for the first light-mediated effect observed by Mester in 1968 (hypertrichosis in mice [2]) but now widely used clinically to restore hair growth in adult humans [129]. Some researchers have hypothesized that this effect was due to polycystic ovarian syndrome present in 5 out of 49 female patients under laser treatment for facial hirsutism, others suggested that even if the heat generated by PBM was not able to ablate cells from the hair follicle, the small amount of heat supposedly produced could induce follicular stem cells to proliferate and differentiate, due to the increased level of heat shock proteins. Another possibility relies on the release of certain factors that could affect the cell cycle and induce angiogenesis [129]. The exact mechanism still needs clarifying, but the effects of PBM on hair growth are already well described.

Hair growth is divided basically in three phases: anagen, catagen and telogen. The anagen is the growth phase and can last from 2 to 6 years. Catagen phase lasts from 1 to 2 weeks and consists of club hair transitions upwards toward the skin pore, while the dermal papilla separates from the follicle. In the telogen phase, the dermal papillae fully separate from the hair follicle. It lasts from 5 to 6 weeks, until the papillae move upward to meet the hair follicles again and the hair matrix begins to form new hair, returning to the anagen phase. It has been observed that PBM is able to stimulate telogen hair follicles to enter the anagen phase, as well as to prolong the duration of the anagen phase itself. PBM is also capable of increasing the rate of proliferation of anagen hair follicles and to prevent premature catagen phase entry. This could be due to induced protein synthesis by the transcription factors activated by PBM, followed by cell migration and proliferation, alteration in cytokines levels, growth factors and inflammatory mediators. NO is also augmented in LLL treated tissues, usually dissociated from Cox, and since it is a well known vasodilator, it is likely that there is a vasodilation effect on hair follicles after PBM that could help hair growth. Some inflammatory mediators also have their expression inhibited by PBM (such as IFN-γ, IL-1a, IL-1b, TNF and Fas-antigen) and, considering that inflammation is highly disruptive for hair follicles, the anti-inflammatory effect of PBM could be useful in the treatment of hair conditions such as alopecia areata [129].

High Fluence Low Power Laser Irradiation (HF-LPLI)

Fluence, according to the International System of Units, is the energy density integrated over the unit surface of a sphere. Just like PBM using low fluences of light, high-fluence low-power laser irradiation (HF-LPLI) stimulates mitochondrial chromophores, but this time it overstimulates them, which in turn activates the mitochondrial apoptosis pathway, altering the cell cycle, inhibiting cell proliferation and even causing cell death. HF-LPLI (usually fluences above 80 J cm^{-2}) induces apoptosis by activating caspase-3, and mitochondrial permeability transition after HF-LPLI is the main mechanism of mitochondrial injury. In 2010, Sun et al. reported that signal transducer and activator of transcription 3 (Stat3) was involved in HF-LPLI-induced apoptosis *in vitro*, and this effect is time- and dose-dependent. Steroid receptor coactivator (Src) seems to be the main upstream kinase of Stat3 activation, and the increased ROS generation plays a key role in this process [130].

Recently, Wu et al. found that HF-LPLI, using light at 633 nm and 120 J cm^{-2}, could ablate tumors via activation of mitochondrial apoptotic pathway after ROS generation. The evidence is based on the inactivation of caspase-8, activation of caspase-9 and by the release of cytochrome C. When this high dose is used, light inactivates Cox (instead of activating Cox), inducing a superoxide burst in the electron transport chain and, finally, produces oxidative damage against cancer cells [29]. Chu and co-workers already observed that PBM could induce a mitochondrial permeability pore transition when higher levels of ROS are produced. As a consequence, the decrease of mitochondrial transmembrane potential causes the permeabilization of the mitochondrial outer membrane and, subsequently, the release of cytochrome c and caspase cascade reaction [131].

Cho also observed the interference that a protein, called survivin, could affect the outcomes of HF-LPLI. Light treatment can activate survivin by inducing an increase in its phosphorylation levels. The activated survivin is able to inhibit the permeabilization of the mitochondrial outer membrane, and therefore prevents the release of cytochrome c, the activation of Bax and caspase-9. Cho then concluded that survivin mediates self protection of tumor cells against HF-LPLI-induced apoptosis, through ROS/cdc25c/CDK1 signaling pathway [131].

Conclusions

Low levels of red/NIR light can interact with cells, leading to changes at the molecular, cellular and tissue levels. Each tissue, however, can respond to this light-interaction differently, although it is well known that the photons, especially in the red or NIR, are predominantly absorbed in the mitochondria [132]. Therefore, it is likely that even the diverse results observed with PBM share the basic mechanism of action. What happens after the photon absorption is yet to be fully described, since many signaling pathways seem to be activated. It seems that the effects of PBM are due to an increase in the

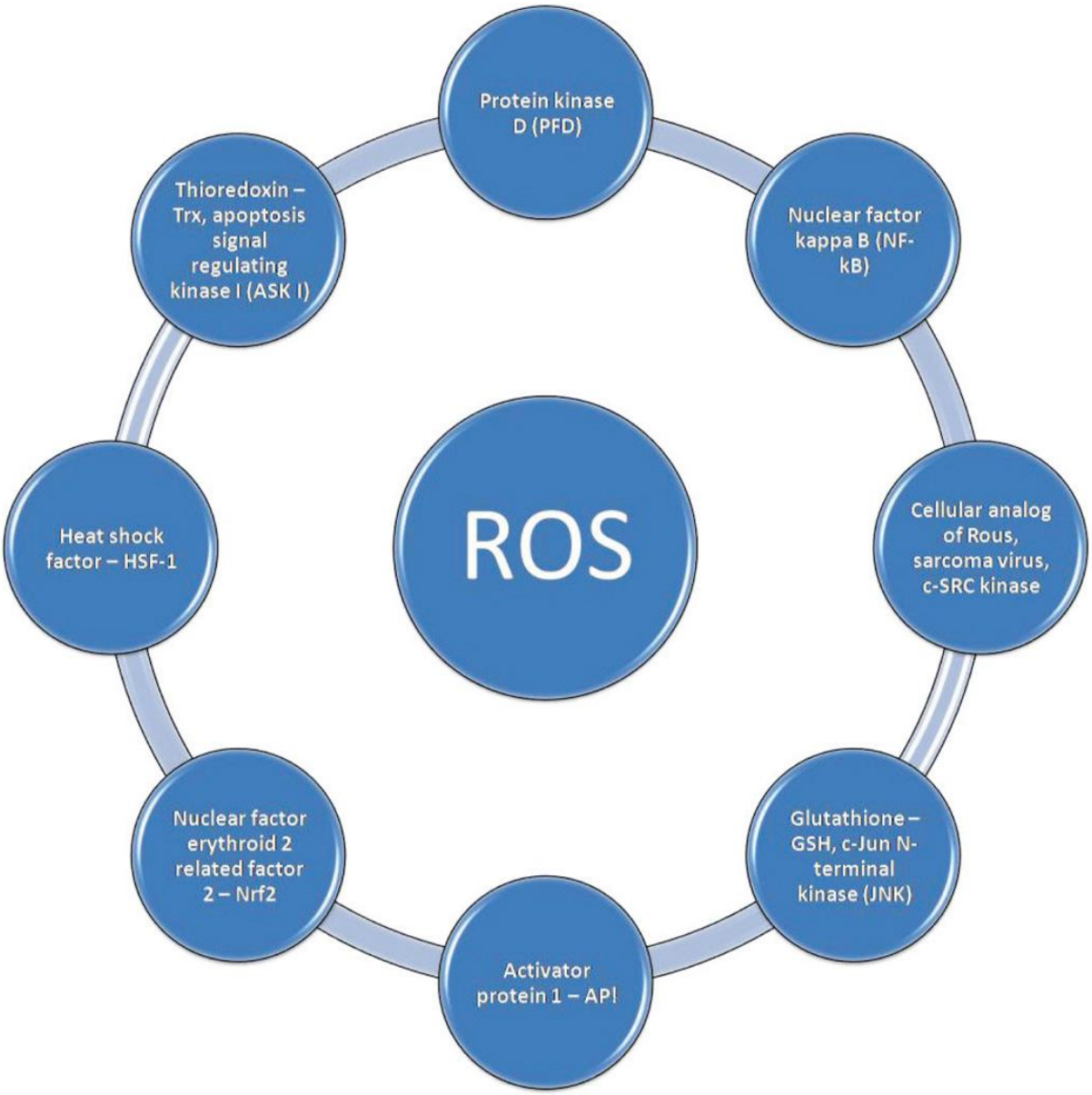
oxidative metabolism in the mitochondria [133]. Different outcomes can occur depending on the cell type, i.e. cancer cells that tend to proliferate when PBM is delivered [88]. In this review we have not discussed the response of cells and tissues to wavelengths longer than NIR, namely far IR radiation (FIR) (3 μm to 50 μm). At these wavelengths water molecules are the only credible chromophores, and the concept of structured water layers that build up on biological lipid bilayer membranes has been introduced to explain the selective absorption [134]. Nevertheless FIR therapy has significant medical benefits that are somewhat similar to those of PBM [135], and it is possible that activation of light/heat sensitive ion channels could be the missing connection between the two approaches.

As we have shown, PBM can regulate many biological processes, such as cell viability, cell proliferation and apoptosis, and these processes are dependent on molecules like protein kinase c (PKC), protein kinase B (Akt/PKB), Src tyrosine kinases and interleukin-8/1a (IL-8/1a). The effects of light on cell proliferation can be stimulatory at low fluences (which is useful in wound healing, for instance), but could be inhibitory at higher light doses (which could be useful in certain types of scar formation such as hypertrophic scars and keloids) [131].

The applications of PBM are broad. Four clinical targets, however, are the most common: shining light on injured sites to promote healing, remodeling and/or to reduce inflammation; on nerves to induce analgesia; on lymph nodes in order to reduce edema and inflammation; and on trigger points (a single one of as many as 15 points) to promote muscle relaxation and to reduce tenderness. Since it is non invasive, PBM is very useful for patients who are needle phobic or for those who cannot tolerate therapies with non-steroidal anti-inflammatory drugs [83].

The positive outcomes depend on the parameters used on the treatment. The anti-inflammatory effect of light in low intensity was reported on patients with arthritis, acrodermatitis continua, sensitive and erythematous skin, for instance [136]. With the same basic mechanism of action, which is the light absorption by mitochondrial chromophores, mainly Cox, the consequences of PBM are various, depending on the parameters used, on the signaling pathways that are activated and on the treated tissue. In order to apply PBM in clinical procedures, the clinicians should be aware of the correct parameters and the consequences for each tissue to be treated. More studies have to be performed in order to fill the gaps that still linger in the basic mechanisms underlying LLLT and PBM.

Figure 5.



Reactive Oxygen Species sensors and signaling

Acknowledgments

MRH was supported by US NIH grant R01AI050875.

Lucas Freitas de Freitas was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP.

Biography

Michael R Hamblin Ph.D. is a Principal Investigator at the Wellman Center for Photomedicine at Massachusetts General Hospital, an Associate Professor of Dermatology at Harvard Medical School and is a member of the affiliated faculty of the Harvard-MIT Division of Health Science and Technology. He was trained as a synthetic organic chemist and received his PhD from Trent University in England. His research interests lie in the areas of photodynamic therapy (PDT) for infections, cancer, and stimulation of the immune system, and in low-level light therapy (LLLT) for wound healing, arthritis, traumatic brain injury, neurodegenerative diseases and psychiatric disorders. He directs a laboratory of around a dozen post-doctoral fellows, visiting scientists and graduate students. His research program is supported by NIH, CDMRP, USAFOSR and CIMIT among other funding agencies. He has published over 320 peer-reviewed articles, over 150 conference proceedings, book chapters and International abstracts and holds 10 patents. He is Associate Editor for 7 journals, on the editorial board of a further 30 journals and serves on NIH Study Sections. For the past 11 years Dr Hamblin has chaired an annual conference at SPIE Photonics West entitled "Mechanisms for low level light therapy" and he has edited the 11 proceedings volumes together with four other major textbooks on PDT and photomedicine. He has several other book projects in progress at various stages of completion. In 2011 Dr Hamblin was honored by election as a Fellow of SPIE. He is a Visiting Professor at universities in China, South Africa and Northern Ireland.

Transcranial laser stimulation: Mitochondrial and cerebrovascular effects in younger and older healthy adults

Authors Celeste L. Saucedo, Emily C. Courtois, Zachary S. Wade, Meghan N. Kelley, Nusha Kheradbin, Douglas W. Barrett, F. Gonzalez-Lima, Mendeley

Highlights

- •
  Transcranial infrared laser is a new and safe method of noninvasive brain stimulation.
- •
  Bilateral prefrontal metabolism was monitored by broadband near-infrared spectroscopy.
- •
  There was a greater laser-induced effect on cytochrome-c-oxidase with increasing age.
- •
  Laser-induced effects on cerebral hemodynamics decreased with increasing age.
- •
  Transcranial infrared laser produced beneficial metabolic effects on the aging brain.

Abstract

Background

Transcranial laser stimulation is a novel method of noninvasive brain stimulation found safe and effective for improving prefrontal cortex neurocognitive functions in healthy young adults. This method is different from electric and magnetic stimulation because it causes the photonic oxidation of cytochrome-c-oxidase, the rate-limiting enzyme for oxygen consumption and the major intracellular acceptor of photons from near-infrared light. This photobiomodulation effect promotes mitochondrial respiration, cerebrovascular oxygenation and neurocognitive function. Pilot studies suggest that transcranial photobiomodulation may also induce beneficial effects in aging individuals.

Objectives

Randomized, sham-controlled study to test photobiomodulation effects caused by laser stimulation on cytochrome-c-oxidase oxidation and hemoglobin oxygenation in the prefrontal cortex of 68 healthy younger and older adults, ages 18–85.

Methods

Broadband near-infrared spectroscopy was used for the noninvasive quantification of bilateral cortical changes in oxidized cytochrome-c-oxidase and hemoglobin oxygenation before, during and after 1064-nm wavelength laser (IR-A laser, area: 13.6 cm^2, power density: 250 mW/cm^2) or sham stimulation of the right anterior prefrontal cortex (Brodmann Area 10).

Results

As compared to sham control, there was a significant laser-induced increase in oxidized cytochrome-c-oxidase during laser stimulation, followed by a significant post-stimulation increase in oxygenated hemoglobin and a decrease in deoxygenated hemoglobin. Furthermore, there was a greater laser-induced effect on cytochrome-c-oxidase with increasing age, while laser-induced effects on cerebral hemodynamics decreased with increasing age. No adverse laser effects were found.

Conclusion

The findings support the use of transcranial photobiomodulation for cerebral oxygenation and alleviation of age-related decline in mitochondrial respiration. They justify further research on its therapeutic potential in neurologic and psychiatric diseases.

- Previous article in issue
- Next article in issue

Keywords

Transcranial infrared laser stimulation
Photobiomodulation
Cytochrome-c-oxidase
Cerebrovascular oxygenation
Brain aging

Introduction

Transcranial infrared laser stimulation (TILS) is a form of low-level laser therapy or photobiomodulation (PBM) [1,2] that uses low-power 1064-nm wavelength laser for noninvasive brain stimulation [3]. The mechanism of action of PBM is different than those of magnetic and electrical brain stimulation [2,3]. While magnetic and electrical stimulation directly modify membrane electrical excitability, in PBM the direct absorption of photons by mitochondria upregulates oxygen consumption and hemodynamics to facilitate brain activity [3,4].

Five recent controlled human studies showed that TILS to the forehead with a 1064-nm wavelength laser photo-oxidizes the mitochondrial enzyme cytochrome-c-oxidase (CCO) and promotes hemoglobin oxygenation in the prefrontal cortex (PFC) [[5], [6], [7], [8]]; these actions were not due to thermal effects [9]. Since neurons are critically dependent on oxygen metabolism, TILS actions on CCO and hemoglobin oxygenation lead to multiple beneficial effects [3,10]. These effects include more efficient oxygen consumption for bioenergetics [2,3], resulting in enhanced PFC-based cognitive functions such as attention and working memory [5,11,12], executive function [13], and rule-based category learning [14].

Transcranial PBM penetrates to the cerebral cortex [15] and acts on CCO because this enzyme is the major intracellular acceptor of photons from red-to-near-infrared light [[16], [17], [18]]. CCO plays key roles in mitochondrial respiration, by catalyzing the reduction of oxygen to water and enabling adenosine triphosphate (ATP) production [19], and in hemodynamics, by catalyzing the synthesis of the vasodilator nitric oxide (NO) under low oxygen conditions [20]. TILS of the PFC augments CCO affinity for oxygen, increasing cerebral oxygen consumption, ATP production, NO release and cerebrovascular oxygenation, which facilitate neurocognitive functions [5,10,16,19].

In young adults, TILS to the PFC induces time-dependent changes in oxidized CCO and hemodynamic activity [6,8]. Concentration changes of oxidized CCO (Δ[CCO]), oxygenated hemoglobin (Δ[HbO]) and deoxygenated hemoglobin (ΔHb]) can be measured *in vivo* by broadband near-infrared spectroscopy (bbNIRS) [21]. This procedure entails shining broadband (white) light into the forehead via optical fibers. Some of this light is absorbed by photoacceptors inside the brain on the basis of their absorption spectrum. Photodetectors are used to measure the light that is scattered and returned to the surface of the head. Fewer photons detected from a particular spectrum of wavelengths indicate a greater concentration of the chromophore with an absorption spectrum at those wavelengths [5].

Using bbNIRS, an 8-min 1064-nm wavelength laser stimulation to the right PFC produced a significant increase in Δ[CCO], followed by an increase in Δ[HbO] and a decrease in Δ[Hb], suggesting that CCO upregulation provokes the increase in hemoglobin oxygenation [8]. Laser-induced effects were not only significant during stimulation, but were maintained throughout a 5-min post-stimulation period. These results were corroborated by another study that revealed the same increase in Δ[CCO] and Δ[HbO] [6]. Sham placebos in these studies showed no significant changes in [CCO], [HbO] or [Hb].

Pilot studies suggest that TILS and other forms of brain PBM may be beneficial in aging adults [6,22,23]. Deficits in cerebral oxygen metabolism and blood flow [[24], [25], [26], [27]] and mitochondrial dysfunction play key roles in aging [[28], [29], [30]]. By catalyzing the reduction of oxygen to water, CCO promotes mitochondrial respiration and limits the formation of reactive oxygen species detrimental to aging neurons [29,31]. Given that the PFC is more susceptible to age-related mitochondrial and cognitive deficits [32,33], TILS of the PFC may be able to alleviate these deficits in older adults. A bbNIRS pilot study of ipsilateral TILS effects that included several older adults showed promising results [6], but there are no bbNIRS studies examining bilateral TILS effects on the PFC in older adults.

The goal of this randomized, sham-controlled study was to use bbNIRS to measure bilateral Δ[CCO], Δ[HbO], and Δ[Hb] during and after PFC laser stimulation in healthy younger and older adults, and to examine age-related differences in response to TILS. Consistent with previous findings, we hypothesized that the laser group would show a significant ipsilateral increase in Δ[CCO] and Δ[HbO], and a significant decrease in Δ[Hb], compared to the placebo group and that these effects would be maintained throughout a 5-min post-stimulation period.

Materials and methods

Participants and study procedures

From the University of Texas at Austin (UT) and Austin community, we enrolled 83 participants, but 15 withdrew due to scheduling conflicts, for a final sample of 68 healthy adult participants (52 females), 18–85 years old, randomized into either laser treatment (n = 35) or placebo/sham (n = 33; Table 1). Randomization was stratified by age (<45 or ≥ 45 years old) and gender (male or female), with a 1:1 treatment:placebo ratio. A total of n = 17 participants (average age: 35.5 years; 12 females; 5 males) were excluded because of technical issues and movement artifacts from the bbNIRS system. Table 2 reports the descriptive statistics for the n = 51 participants included in the analysis.

Table 1. Participant demographics by condition.

Demographic	Laser (n = 35)	Placebo (n = 33)	*p*-value[a]
Age (years)			0.76
Mean (SD)	35.7 (20.0)	34.2 (19.6)	
Median [min, max]	25.0 [18.0, 85.0]	22.0 [18.0, 73.0]	
Younger (< 45 years)	*(n = 25)*	*(n = 23)*	
Mean (SD)	24.2 (6.5)	22.3 (5.3)	
Median [min, max]	21.0 [18.0, 43.0]	21.0 [18.0, 38.0]	
Older (≥ 45 years)	*(n = 10)*	*(n = 10)*	
Mean (SD)	64.7 (10.0)	61.7 (9.6)	
Median [min, max]	63.5 [50.0, 85.0]	60.0 [48.0, 73.0]	
Gender			0.88
Female	26 (74.3%)	26 (78.8%)	
Male	9 (25.7%)	7 (21.2%)	
Race			0.38
White	25 (71.4%)	20 (60.6%)	
Black/African-American	1 (2.9%)	3 (9.1%)	

Demographic	Laser (n = 35)	Placebo (n = 33)	*p*-value[a]
Asian	6 (17.1%)	9 (27.3%)	
Other	3 (8.6%)	1 (3.0%)	
Ethnicity			1
Not Hispanic/Latino	29 (82.9%)	28 (84.8%)	
Hispanic/Latino	6 (17.1%)	5 (15.2%)	
Education			0.35
High School	21 (60.0%)	18 (54.5%)	
Bachelor's Degree	6 (17.1%)	6 (18.2%)	
Master's Degree	4 (11.4%)	8 (24.2%)	
Professional Degree	4 (11.4%)	1 (3.0%)	
Global Cognition Score (MoCA)[b]			0.23
Mean (SD)	26.8 (2.59)	27.4 (1.60)	
Median [min, max]	27.0 [19.0, 30.0]	28.0 [25.0, 30.0]	
Younger (< 45 years)	*(n = 25)*	*(n = 23)*	
Mean (SD)	27.0 (2.2)	27.4 (1.8)	
Median [min, max]	28.0 [22.0, 30.0]	27.0 [25.0, 30.0]	
Older (≥ 45 years)	*(n = 10)*	*(n = 10)*	
Mean (SD)	26.2 (3.5)	27.5 (1.3)	
Median [min, max]	27.0 [19.0, 30.0]	28.0 [26.0, 30.0]	

p-value > 0.05 indicates demographic characteristic did not differ significantly between groups.

a Chi-squared tests were used for categorical variables and t-tests were used for continuous variables.

b MoCA, Montreal Cognitive Assessment. Score out of 30 (≥26 = normal cognition).

Table 2. Demographics of participants included in statistical analyses.

Demographic	Laser (n = 23)	Placebo (n = 28)	*p*-value[a]
Age (years)			0.72
Mean (SD)	36.0 (20.5)	34.0 (20.4)	
Median [min, max]	25.0 [18.0, 85.0]	22.0 [18.0, 73.0]	
Younger (< 45 years)	*(n = 17)*	*(n = 20)*	
Mean (SD)	25.0 (7.2)	22.0 (5.4)	
Median [min, max]	21.0 [18.0, 43.0]	20.5 [18.0, 38.0]	

Demographic	Laser (n = 23)	Placebo (n = 28)	*p*-value[a]
Older (≥ 45 years)	*(n = 6)*	*(n = 8)*	
Mean (SD)	67.3 (10.1)	64.0 (9.4)	
Median [min, max]	64.0 [56.0, 85.0]	66.0 [48.0, 73.0]	
Gender			0.71
Female	17 (73.9%)	23 (82.1%)	
Male	6 (26.1%)	5 (17.9%)	
Race			0.35
White	19 (82.6%)	17 (60.7%)	
Black/African-American	1 (4.3%)	2 (7.1%)	
Asian	3 (13.0%)	8 (28.6%)	
Other	0 (0%)	1 (3.6%)	
Ethnicity			1
Not Hispanic/Latino	18 (78.3%)	23 (82.1%)	
Hispanic/Latino	5 (21.7%)	5 (17.9%)	
Education			0.79
High School	15 (65.2%)	17 (60.7%)	
Bachelor's Degree	4 (17.4%)	6 (21.4%)	
Master's Degree	2 (8.7%)	4 (14.3%)	
Professional Degree	2 (8.7%)	1 (3.6%)	
Global Cognition Score (MoCA)[b]			0.09
Mean (SD)	26.6 (2.98)	27.8 (1.51)	
Median [min, max]	27.0 [19.0, 30.0]	28.0 [25.0, 30.0]	
Younger (< 45 years)	*(n = 20)*	*(n = 17)*	
Mean (SD)	27.7 (1.7)	27.1 (2.4)	
Median [min, max]	28.0 [22.0, 30.0]	28.0 [25.0, 30.0]	
Older (≥ 45 years)	*(n = 6)*	*(n = 8)*	
Mean (SD)	25.2 (4.2)	27.9 (1.1)	
Median [min, max]	27.0 [19.0, 30.0]	28.0 [26.0, 30.0]	

p-value > 0.05 indicates demographic characteristic did not differ significantly between groups.

a Chi-squared tests were used for categorical variables and t-tests were used for continuous variables.

b

MoCA, Montreal Cognitive Assessment. Score out of 30 (≥26 = normal cognition).

Participants were excluded if they were either pregnant, had a history of violent behavior, had ever been institutionalized or imprisoned, had been previously diagnosed with a major neurological disorder (i.e., stroke, traumatic brain injury, Parkinson's disease), major psychiatric disorder (i.e., bipolar disorder, personality disorder, schizophrenia, obsessive compulsive disorder), major or mild neurocognitive disorder, or had been recently diagnosed with alcohol or drug abuse, severe anxiety or depression.

All procedures (Table 3) were approved by the University of Texas at Austin's Institutional Review Board. Eligible participants signed an informed consent form and completed a medical history questionnaire. Six participants reported hypertension and two reported diabetes, which could potentially affect the cerebrovascular response to TILS; however, these conditions were being controlled with medication, and participants were not excluded on this basis.

Table 3. Experimental design.

Week 1
1. Informed consent
2. Medical history questionnaire
3. MoCA
Week 2
4. Randomization
5. bbNIRS session + laser/sham procedure
6. Side effects?
7. Debriefing
8. Compensation

The Montreal Cognitive Assessment (MoCA) was administered to measure global cognitive functioning to gauge baseline cognitive ability and ensure there were no significant group differences (laser vs. placebo). The highest score that can be obtained is 30, and a score ≥26 is considered normal cognition [34]. Neither the MoCA nor self-report regarding cognitive functioning are sensitive to subtle cognitive or executive dysfunction. Thus, the MoCA was not intended to be used for diagnostic purposes or quantification of participants' cognitive changes. While lower MoCA scores are generally found in older participants, wide variability in MoCA scores may be also due to cross-cultural limitations in how this measure is applied [35]. These limitations are part of the reasoning behind not using the MoCA for diagnostic purposes or as a dependent variable indicating participants' cognitive ability.

One week later (±2 days), participants were randomized into laser or placebo groups with a 1:1 treatment:placebo stratified ratio and completed a bbNIRS session. All participants were asked about side effects (no adverse effects from the laser treatment

were reported). At the end of the experiment, participants were debriefed on their group assignment and were given monetary compensation for their participation.

Brain stimulation

TILS was applied for 8 min to the right forehead (Fig. 1) using a continuous wave (1064 nm wavelength) laser procedure that increases CCO and hemodynamic responses [8] and cognition in young adults [3]. TILS was aimed at the right anterior PFC (Brodmann Area 10; Fig. 2), centered on point FP2 as defined by the 10–20 EEG coordinate system. A previous TILS study using the same laser parameters showed that right (but not left) PFC stimulation was behaviorally effective [36]. The placebo group underwent a sham 5-s stimulation during the first minute, which does not generate physiological or cognitive effects [8,11] but gives a similar subjective experience to the TILS group. Since a slight warm sensation at the beginning of the laser treatment is commonly reported, the brief 5-s stimulation period was used to induce a similar subjective experience between the placebo and treatment groups and ensure participants remained blind to their group assignment. During the 55 s of no treatment, the laser light was turned off, but the apparatus was left turned on so that all participants were exposed to the sound that the laser apparatus emits when in use. All participants (treated and sham) wore dark goggles and were instructed to keep their eyes closed throughout the procedure. This sham procedure was proven to be effective in previous research because participants could not correctly report whether they got sham or active treatment [11]. Each laser and sham procedure was done inside a locked laser room with a sign outside the door indicating that the laser was in use. Participants and experimenters wore protective eyewear (900–1000 nm: 5+, 1000–2400 nm: 7+; 2900–10600 nm: 7+) at all times.

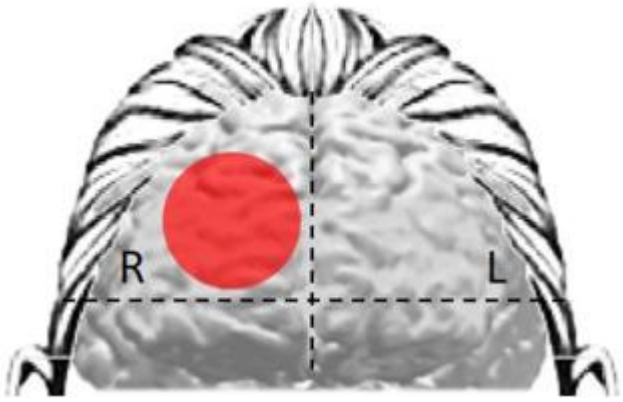


Fig. 1. Laser treatment procedure. Laser treatment consisted of 8 1-min laser stimulations (red) administered to the right PFC (Brodmann Area 10). The control group received a sham treatment, which consisted of a brief 5-s stimulation during the first minute of the procedure only. R, participant's right hemisphere; L, participant's left hemisphere. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

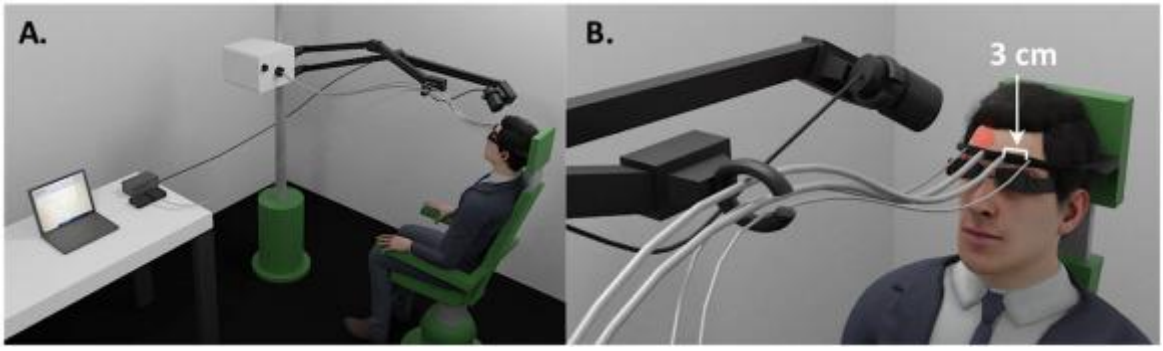


Fig. 2. bbNIRS system and setup. A) Source wires (thick) from light box (white) provided broadband light to the forehead. Detector wires (thin) were hooked up to spectrophotometer boxes (black) where concentration changes were measured. B) Laser was aimed to right side of forehead and head was fastened to the chair using a leather strap. The laser was placed 2 inches from forehead.

Laser apparatus and parameters

The experiment used a 1064-nm wavelength (infrared IR-A category, also called near infrared) laser device (CytonPro 5000, CytonSys, Inc., Austin, TX, USA). While photon absorption by CCO has peaks closer to 660 nm and 810 nm and absorption decreases as the wavelength approaches 1064 nm [37], light scattering is more common at shorter wavelengths, which prevents light from traveling too deeply through tissue. Monte Carlo simulations for photon delivery into the PFC with three representative wavelengths (660 nm, 810 nm and 1064 nm) showed that 1064 nm is the optimal, benefiting from its reduced tissue scattering [38]. Other photoacceptors such as water also absorb photons in both the lower and higher wavelengths in this range. The longer 1064-nm wavelength allows for deeper tissue penetration and less scattering, so there is a trade-off between these two variables (absorption of light by CCO and depth of penetration).

The collimated beam was circular and covered 13.6 cm^2. The measured output was 3.4 W; therefore, the treated region was exposed to a power density of 3400 mW/13.6 cm^2 = 250 mW/cm^2 for 8 min for a total of 3.4 W × 480 s = 1632 J and an energy density of 0.25 W/cm^2 x 480 s = 120 J/cm^2. These are the same parameters that have shown psychologically beneficial effects in previous studies [11,13,14,39]. The sham condition received 3.4 W × 5 s = 17 J for a total energy density of 0.25 W/cm^2 x 5 s = 1.25 J/cm^2 (<1% of the energy density used in the laser group). The laser and standard operating procedure were reviewed and approved by the University of Texas at Austin's Laser Safety Office.

Broadband near-infrared spectroscopy (bbNIRS)

A non-invasive, two-channel bbNIRS system (Fig. 2) was custom-made to measure laser-induced changes in concentrations of CCO (Δ[CCO]), oxygenated hemoglobin (Δ[HbO]), and deoxygenated hemoglobin (Δ[Hb]), as indices of mitochondrial and hemodynamic changes in the PFC. We have investigated the sensitivity and specificity of

this bbNIRS method to detect TILS-induced brain effects as opposed to that of connective tissue or skin. When compared using CCO/hemodynamic ratios, superficial tissues (measured at 1.5 cm emitter-detector separation) showed approximately 5% above baseline whereas brain changes (measured at 3 cm emitter-detector separation) showed approximately 20% (Fig. 5 of Wang et al., 2017 [8]). Therefore, this bbNIRS method does not seem to receive a heavy contribution from superficial tissues.

This system consisted of a light source (tungsten-halogen lamp with a spectral range of 400–1500 nm), spectrophotometers (spectral range 735–1100 nm), spectrophotometer cables (detectors), and optical fiber bundles (light emitters). The light source (Model 3900, Illumination Technologies, Inc., East Syracuse, NY, USA) provided broadband light to the forehead via the emitters. Detectors relayed light that was reflected back to the surface of the head to the spectrophotometers (QE-Pro, Ocean Optics, Inc., Dunedin, FL, USA). One channel, comprised of one emitter (applying light to the forehead) and one detector (detecting light reflected back), took measurements of Δ[CCO], Δ[HbO], and Δ[Hb] from the right hemisphere (ipsilateral to the stimulation site), and the other channel took measurements from the left hemisphere (contralateral to the stimulation site). A low-pass filter with a cutoff frequency of 1000 nm was placed at the base of the emitters to avoid overlap between the broadband light and the laser and reduce heating. Broadband light intensity was set at the device's lowest level (40 mW) as measured during sessions using a Newport optical meter (Model 1919-R, Irvine, CA, USA). This light was safe and did not cause any heat-related tissue damage or discomfort to the participant.

Before being hooked up to the stimulation and measuring setup, participants were walked through the setup in detail. They were instructed to remain as still as possible and to refrain from clenching their jaw, moving their eyelids, and readjusting their body, as movement would introduce artifacts in the data. They were also instructed to refrain from crossing their arms or legs, since this would affect blood circulation. Then participants were asked to sit comfortably at the bbNIRS chair, which was an adjustable optometrist chair. An elastic strap was placed snugly around the participant's head right above the eyebrows to hold the probes in place and leave the rest of the forehead exposed for laser stimulation (Fig. 2B). An additional leather strap was placed on top of the elastic strap in order to secure the participant's head against the headrest and minimize head movement. Each probe had two openings 3-cm apart for the emitter and detector cables. Comfort level was monitored throughout the setup and adjustments were made accordingly. Participants were asked to close their eyes and keep them closed throughout the session. The laser was positioned at a distance of 2 inches.

A laptop computer with OceanView software (Version 1.6.7, Ocean Optics, Inc., Dunedin, FL, USA) was used to acquire, display, and save the recordings of the power spectrum of the light returning to the surface of the head. This power spectrum was used to determine changes in concentration of CCO, HbO, and Hb during the laser/sham procedure. The spectrum and photon count displayed on the screen was updated every 5 s. To avoid potential contamination of the power spectrum during the laser procedure, an interleaved method was used during the 8 1-min laser treatments (Fig. 3); the laser light was turned off after each minute of stimulation to allow for a 5-s period of bbNIRS data acquisition in the absence of laser stimulation [7,8]. Once a stable baseline of

spectra and photon count was established, two baseline recordings were obtained 1 min apart. Immediately after the second baseline recording, the laser was turned on to begin the laser procedure (or the brief 5-s stimulation in the sham procedure), and the interleaved method was used for the next eight recordings. Finally, five more recordings were obtained after the laser (or sham) procedure, 1 min apart. A total of 15 recordings were obtained per participant, one per minute.

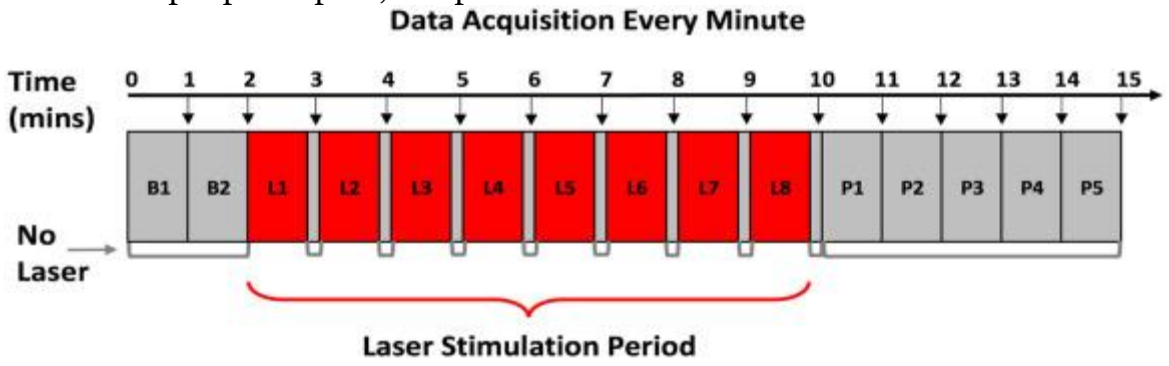


Fig. 3. bbNIRS session interleaved method for data acquisition. Readings were acquired every minute (indicated by the down arrows). B1–B2, 2 min of baseline; L1–L8, 8 min of laser stimulation. Gray areas after each min indicate laser-off period to acquire readings. P1–P5, 5 min post-stimulation when the laser was off (recordings were acquired every min).

Data processing and statistics

The bbNIRS data was pre-processed using MATLAB (MathWorks, Natick, MA, USA), and statistical analyses were conducted using R Studio. Changes in chromophore concentrations (i.e., Δ[CCO], Δ[HbO], Δ[Hb]) were calculated with the same regression algorithms used in Ref. [40]. Baseline values were used to calculate relative concentration changes at 13 time points (8 min of laser/sham and 5 min of post-laser/sham), evaluated per channel (right and left PFC) for each participant. Seventeen participants with movement or hardware issues were excluded from statistical analyses. Outliers (scores ± 2.5 SD from the group mean at each minute) were identified for each condition (laser and placebo) per chromophore per channel and were also excluded from statistical analyses. Data was detrended from linear trends unrelated to laser-induced effects by regression of the best-fitting line from the placebo group. The best-fitting line was obtained from a regression with time series (minutes) as the predictor and changes in chromophore concentration as the outcome variable. Final, detrended, temporal values for right PFC Δ[HbO] (n = 49), Δ[Hb] (n = 47), and Δ[CCO] (n = 45) and left PFC Δ[HbO] (n = 45), Δ[Hb] (n = 48), and Δ[CCO] (n = 46) were then averaged across all participants under each condition.

An omnibus test using an analysis of variance (ANOVA) with all variables was first used to identify significant main effects and interactions. Further ANOVAs tested each chromophore for all subjects and separately for younger (<45 years) versus older (≥45 years) participants. Two-sample t-tests compared laser and placebo groups for each of the 13 time points. To investigate age-related differences in laser-induced physiological effects, the sample was split into four groups: younger laser and younger placebo (<45

years), and older laser and older placebo (≥45 years). Follow-up regressions were conducted separately for the laser and placebo groups (right and left channels combined) to investigate age as a predictor of the physiological outcomes. Two-tailed $p < 0.05$ was considered significant.

Results

Overall significant main effects and interactions

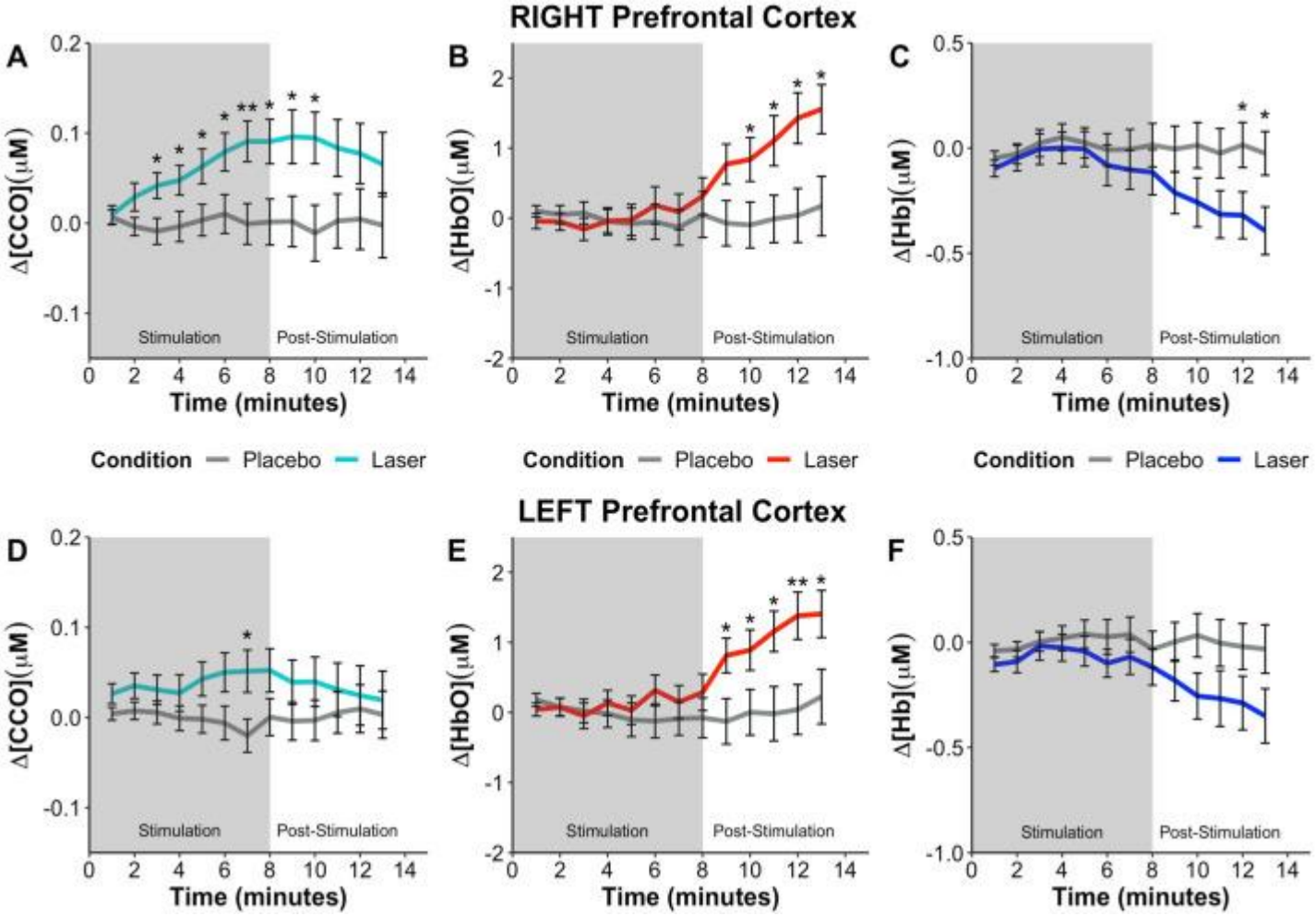
An omnibus ANOVA with all variables identified significant main effects ipsilateral to the laser stimulation region (right PFC) but not contralateral (left PFC). Significant ipsilateral effects included a main effect of condition on right Δ[CCO], $F(1, 42) = 4.86$, $p < 0.05$, and a significant interaction of condition by time on right Δ[HbO], $F(2.25, 103.37) = 6.11$, $p < 0.01$. Further ANOVA testing identified that older participants (≥45 years) contributed the significant effects on Δ[CCO], $F(1, 11) = 5.49$, $p < 0.05$, and on Δ[Hb], $F(12, 120) = 3.56$, $p < 0.001$. In contrast, the younger (<45 years) participants contributed the significant effect on Δ[HbO], $F(2.41, 84.43) = 5.02$, $p < 0.01$.

Comparisons of laser and sham placebo groups

Concentration changes of CCO, HbO, and Hb in the laser and placebo group at 13 time points (mean ± SEM) are illustrated in Fig. 4. There were statistically significant differences ($p < 0.05$ and $p < 0.01$) between the laser and placebo groups in Δ[CCO], Δ[HbO], and Δ[Hb] in an apparent time-dependent manner, a phenomenon previously shown by Wang et al. [8]. In the *right* PFC, ipsilateral to the stimulation, a laser-induced increase in Δ[CCO] was shown primarily during the laser stimulation period (minutes 3–8) and maintained through the second minute of post-stimulation (Fig. 4A). This was followed by a significant increase in Δ[HbO] ($p < 0.05$) starting on the first minute of

post-stimulation (Fig. 4B) and a subsequent decrease in Δ[Hb] ($p < 0.05$) starting the fourth minute post-stimulation (Fig. 4C).

Fig. 4. Comparisons of changes in [CCO], [HbO], and [Hb] between laser and placebo group. In the *right* PFC (ipsilateral to stimulation), Δ[CCO] showed an effect primarily during laser stimulation and Δ[HbO] and Δ[Hb] primarily during the post-stimulation



period. In the left PFC (contralateral), laser-induced changes were seen mainly in Δ[HbO] in the post-stimulation period. Two-sample t-tests were used for the comparisons between the laser and placebo groups at each minute of the session. Significance levels are indicated by $*p < 0.05$, $**p < 0.01$.

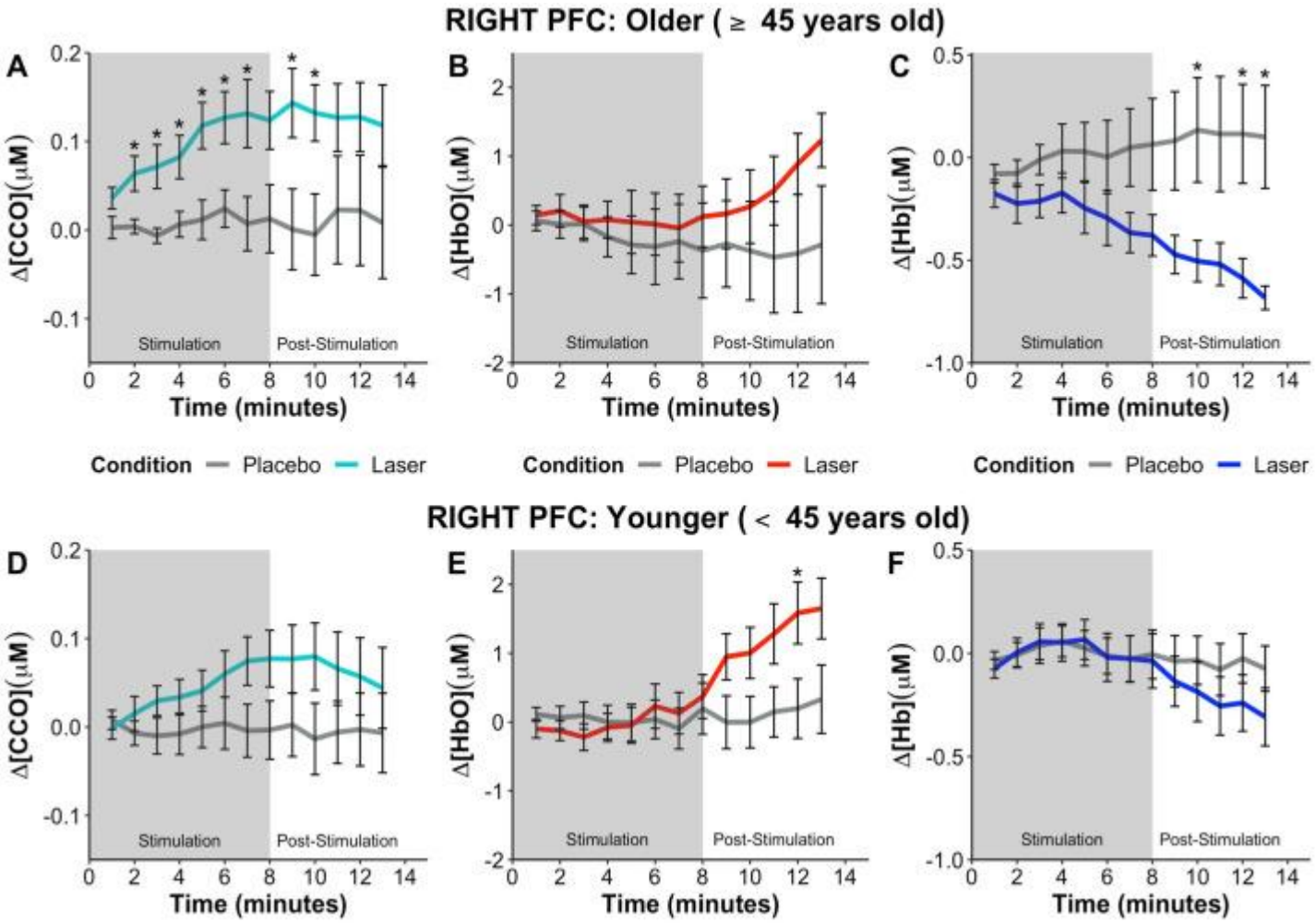
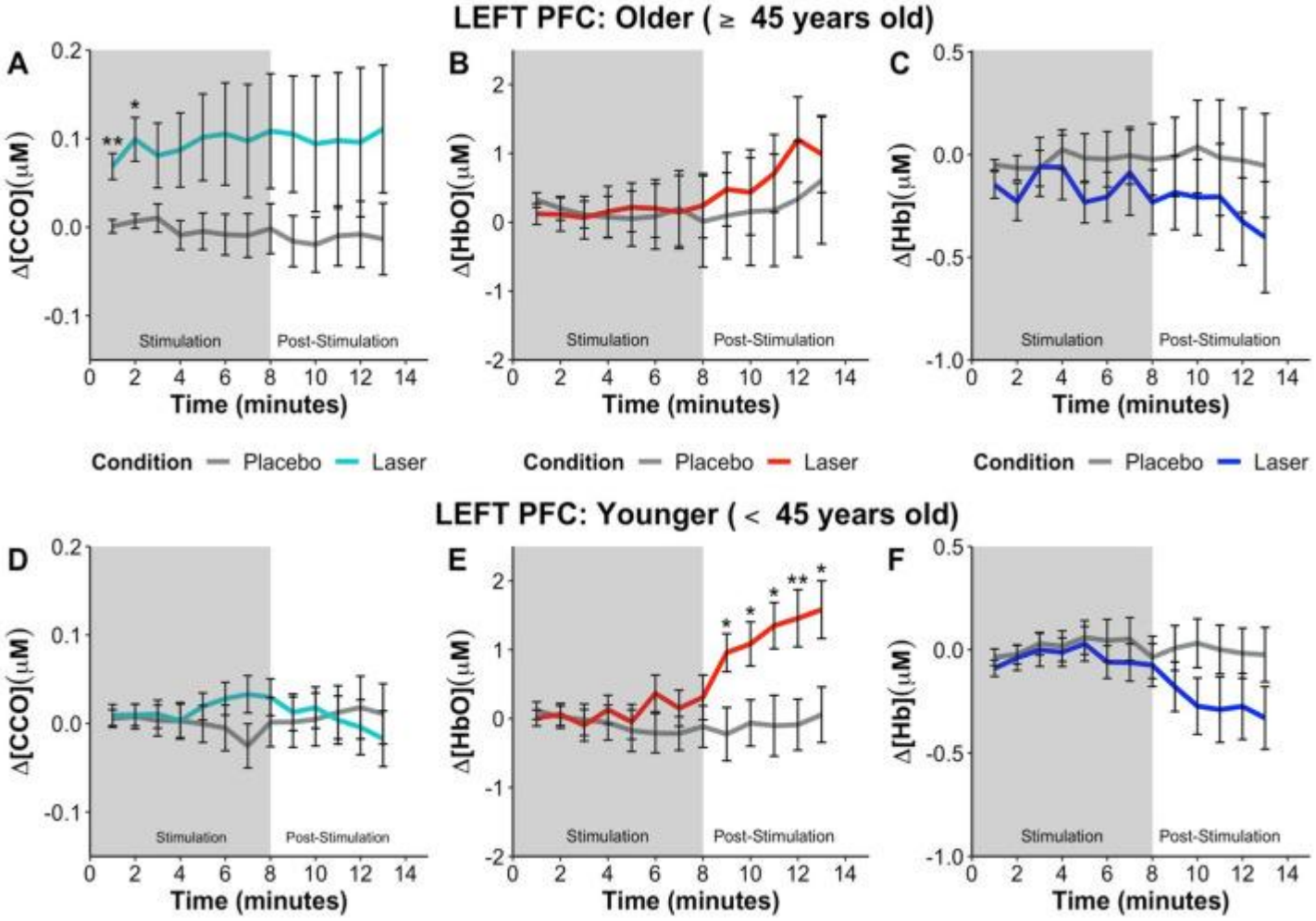


Fig. 5. Right PFC (ipsilateral to stimulation) laser-induced changes in [CCO], [HbO], and [Hb] in younger and older adults. Laser-induced effects were primarily shown in the older group (≥45 years). There was a significant laser-induced increase in concentration of CCO mainly during laser stimulation and a significant laser-induced decrease in concentration of Hb post-stimulation. The younger group (<45 years) showed a brief but significant laser-induced increase in [HbO] during minute four of post-stimulation. Two-sample t-tests were used for the comparisons between the laser and placebo groups at each minute of the session. Significance levels are shown by $*p < 0.05$.

In the *left* PFC, contralateral to the site of stimulation, significant differences between the laser and placebo groups were predominantly shown in Δ[HbO] (Fig. 4E). Interestingly, the pattern of Δ[HbO] in the left PFC mirrors the laser effect shown in the right PFC, also lasting throughout the entire post-stimulation period. As for Δ[CCO] and Δ[Hb], the contralateral trends were weaker with fewer significant differences between the laser and placebo groups (Fig. 4D and F).

Comparisons of younger and older adults

Effects of laser stimulation in younger participants (<45 years old) and older participants (≥45 years old), compared to their respective placebo groups, are illustrated in Fig. 5, Fig. 6. Changes in Δ[CCO] were significantly different ($p < 0.05$ and $p < 0.01$) between the older laser and older placebo groups, primarily in the right PFC, the site of stimulation. A significant laser-induced increase in Δ[CCO] ($p < 0.05$) was shown primarily during the laser stimulation period (minutes 2–7) and maintained through the second minute of post-stimulation (Fig. 5A). This was followed by a significant decrease in Δ[Hb] ($p < 0.05$) starting on the second minute of post-stimulation and maintained for the fourth and fifth minute of post-stimulation (Fig. 5C). There were no significant differences in Δ[HbO] between laser and placebo in the older group (Fig. 5B). In younger participants, the laser group showed similar but weaker trends, with the exception of an increase in right PFC Δ[HbO], which became significantly greater than the placebo ($p < 0.05$) during the fourth minute of post-stimulation (Fig. 5E). However, laser-induced changes in Δ[CCO] and Δ[Hb] did not reach statistical significance



(Fig. 5D and F).

Fig. 6. Left PFC (contralateral to stimulation) laser-induced changes in [CCO], [HbO], and [Hb] in younger and older adults. Laser-induced effects were primarily shown by the younger group (<45 years), which showed a significant increase in HbO concentration during post-stimulation. The older group (≥45 years) showed a brief but significant laser-induced increase in CCO concentration during the first 2 min of laser stimulation. Two-sample t-tests were used for the comparisons between the laser and placebo groups at each minute of the session. Significance levels are shown by $*p < 0.05$, $**p < 0.05$.

In the left PFC, contralateral to the site of stimulation, there was more variability, but changes in Δ[CCO] were significantly different ($p < 0.05$ and $p < 0.01$) between the older laser and older placebo groups during minutes 1–2 of stimulation only (Fig. 6A). There were no significant differences in Δ[HbO] and Δ[Hb] between laser and placebo in the older group in left PFC (Fig. 6B and C). In the younger group, the laser group showed similar but weaker trends, with the exception of a significant laser-induced increase in the left PFC Δ[HbO] ($p < 0.05$ and $p < 0.01$) during the entire post-stimulation period (Fig. 6E). There were no significant laser-induced changes in Δ[CCO] or Δ[Hb] (Fig. 6D and F).

Results from linear regressions using age as a predictor of the physiological outcomes are summarized in Table 4. For the laser group, age was a significant predictor of changes in concentration of CCO, HbO and Hb. More specifically, the laser-induced changes in Δ[CCO] were greater with increasing age ($\beta = 0.001$, $p < 0.01$), while the laser-induced changes in Δ[HbO] ($\beta = -0.011$, $p < 0.05$) and Δ[Hb] ($\beta = -0.007$, $p < 0.001$) diminished with increasing age.

Table 4. Age as a predictor of physiological outcome.

A. Laser Group									
		[CCO]			[HbO]			[Hb]	
Predictor	*Est.*	*CI*	*p*	*Est.*	*CI*	*p*	*Est.*	*CI*	*p*
(Intercept)	0.067	0.054–0.080	**<0.001**	0.484	0.378–0.589	**<0.001**	−0.150	−0.200–0.100	**<0.001**
Age	0.001	0.000–0.002	**0.001**	−0.006	−0.011–0.001	**0.022**	−0.007	−0.010–0.004	**<0.001**
R^2/R^2 adjusted	0.040/0.037			0.010/0.008			0.080/0.077		
B. Placebo Group									
		[CCO]			**[HbO]**			**[Hb]**	
Predictor	*Est.*	*CI*	*p*	*Est.*	*CI*	*p*	*Est.*	*CI*	*p*
(Intercept)	−0.000	−0.013–0.013	1.000	0.000	−0.105–0.105	1.000	−0.000	−0.048–0.048	1.000
Age	0.001	−0.000–0.001	0.119	0.004	−0.001–0.010	0.110	−0.000	−0.003–0.002	0.918
R^2/R^2 adjusted	0.008/0.005			0.004/0.002			0.000/-0.003		

A. Laser stimulation enhanced [CCO] as a function of age (i.e., there was a greater increase in [CCO] with older age), while the hemodynamic responses (e.g., [HbO] and [Hb]) diminished with

increasing age. **B.** As expected, there was no significant effect of age on relative changes in concentration of physiological outcomes in the placebo group.

Discussion

This study, to our knowledge, is the first to investigate the bilateral effects of PBM on brain mitochondrial and hemodynamic activity in healthy younger and older adults. The aging brain experiences significant decline in mitochondrial function, cerebral blood flow, and energy production [[24], [25], [26], [27],33,41,42]. Consequently, we examined differences in laser-induced effects between younger and older individuals. There is growing evidence from studies using bbNIRS and functional NIRS (fNIRS) that our specific TILS of the PFC in healthy adults significantly increases CCO photonic oxidation and cerebral oxygenation [[5], [6], [7], [8]]. The reliability of our bbNIRS method was first shown by Wang et al., 2017 [8] and then by the replication study of Pruitt et al., 2020 [6] in young and elderly participants. We have also shown the hemodynamic increases in [HbO] after TILS during two cognitive tasks (psychomotor vigilance task and delayed match to sample task) in Holmes et al., 2019 [5]. Kolyva et al., 2012 [37] have also previously shown increase on Δ[CCO] from a cognitive task. The current study extends the literature providing sham-controlled evidence that laser stimulation can significantly enhance mitochondrial and hemodynamic effects on the PFC in older humans. Importantly, we revealed novel information about the relationship between age and physiological response to this type of brain stimulation.

Results from the physiological measures supported our hypothesis that the laser group would show a significant increase in Δ[CCO] and Δ[HbO], and a significant decrease in Δ[Hb], compared to the sham placebo group. As expected, a stronger effect of the laser treatment appeared in the right PFC, the site of stimulation. Consistent with previous findings [8], laser-induced effects were time-dependent such that a significant increase was first observed in Δ[CCO] during laser stimulation, with a subsequent increase in Δ[HbO] and decrease in Δ[Hb] during post-stimulation. The previous report by Wang et al. [8] did not show simultaneous changes, but rather sequential changes separated by minutes. Specifically, it showed first an increase in Δ[CCO], followed by increase in Δ[HbO] and then decrease in Δ[Hb]. In the present study, we observed the same sequence of changes: first increase in Δ[CCO] but followed with a more delayed increase in Δ[HbO] and then decrease in Δ[Hb]. The same sequential pattern with a delayed hemodynamic time course may be due to differences in experimental design, apparatus, or our subject population that included older adults.

Though considerably weaker, some significant laser-induced effects were also evident in Δ[CCO] and Δ[HbO] in the left PFC (contralateral to stimulation site). This carryover of laser-induced effects to the left PFC is not surprising given the inter-hemispheric connections via the corpus callosum, and similar contralateral effects have been shown before in a laser-fNIRS study of young participants using the same laser parameters [7]. Our hypothesis that the effects would last for 5 min post-stimulation was partially supported. While the increase in Δ[HbO] did remain significant for the entire post-stimulation, the increase in Δ[CCO] and decrease in Δ[Hb] remained significant during a fraction of the post-stimulation period.

Importantly, our results revealed that age was a significant factor in treatment response. The laser-induced increase in Δ[CCO] and decrease in Δ[Hb] were predominantly seen in older individuals while the increase in Δ[HbO] occurred mainly in younger individuals. Further analyses confirmed that changes in [CCO] were greater with increasing age while changes in [HbO] and [Hb] diminished in older age. Mitochondrial dysfunction is theorized to play a key role in the aging process, particularly the deficiency in oxidative energy metabolism and oxidative damage caused by the imbalance between reactive oxygen species production and antioxidant activity [28]. Our findings showed that laser brain stimulation could successfully increase levels of oxidized CCO, the active form of this enzyme working to reduce oxygen to water [8]. A greater CCO effect in older than in younger participants fuels the speculation that older, metabolically-compromised individuals may benefit more from the laser treatment at a metabolic level, but further investigation will be needed to test this notion.

On the other hand, the greater improvement in oxygenated hemoglobin in younger than in older participants suggests a better hemodynamic response in younger participants. This also suggests that substantial augmentation of oxidized CCO may not be needed in healthy younger participants, with a presumably healthy mitochondrial respiratory system, to provoke a laser-induced surge in oxygenated hemoglobin. A comparable significant increase in oxygenated hemoglobin was not seen in older individuals, though they showed a similar upward trend at the end of the post-stimulation period (Fig. 5B). It is possible that older participants had slower hemodynamic responses, and that their upward trend may have eventually reached significance levels past 5 min of post-stimulation. In fact, the only other study that has investigated the effects of transcranial PBM in Δ[CCO] and Δ[HbO] in younger (n = 15) and older (n = 5) participants [6] found a significant laser-induced ipsilateral increase in Δ[CCO] and Δ[HbO] in younger and older individuals, though Δ[CCO] was lower in the older group. As opposed to the current study, however, they did not have separate sham control groups of younger and older participants; rather, each participant served as their own control by undergoing sequential sham and laser procedures, which cannot rule out a potential order effect on their results. The differences in experimental design and sample size between their study and the current study could be driving some of the differences in results. It is possible that a larger sample of older adults would have revealed the age-dependent laser effects we found. Nonetheless, two findings appeared clear from both studies: 1) TILS can augment Δ[CCO] and Δ[HbO] in younger and older participants, and 2) this treatment is unlikely to be a one-size-fits-all approach, so the individualization of treatment will ultimately be necessary. The ability to increase oxidized CCO concentration in older participants is crucial for improved mitochondrial function and it is an important first step suggesting TILS' ability to alleviate age-related cognitive decline. Thus, findings from the current study are highly encouraging.

Limitations

There were a few limitations while using bbNIRS. Measurements cannot be taken continuously throughout the laser treatment (hence the 5-s-off periods during the laser treatment for bbNIRS measurements) as the laser light would contaminate the bbNIRS detectors. Additionally, the readings were taken from brain regions that are next to, and

not directly under, the laser stimulation site, as the removal of the emitter/detector apparatus from the forehead would be impractical and would invalidate the relative concentration change measurements. The modified Beer-Lambert Law, used to convert light intensities to concentration changes, is reliant on initial baseline measurements, and thus can only return changes in concentration *relative* to the within-session baseline, not *absolute* concentrations. This limitation prevents the accurate across-session comparison of concentration differences. Moreover, while it would be immensely beneficial to obtain recordings past 5 min of post-stimulation, it is difficult to keep recording as participants could fall asleep or become fatigued, affecting the quality of the data. The system is very sensitive to movement-related artifacts, which tend to appear more frequently with increasing procedure time, and these artifacts interfere with data collection and analysis. The bbNIRS data from several participants was lost due to movement or hardware issues, which resulted in smaller and uneven age groups, restricting the statistical analyses looking at the effects of age. This study did not have enough subjects reporting cardiovascular risk factors to control for these factors statistically, but future studies could use enough participants to include these factors as additional independent variables, to see if they may play a role in the physiological response to TILS. The use of the MoCA as a tool to gauge baseline cognitive ability is limited because MoCA is only a screening tool, and the effects of the intervention may be better detected with more sensitive cognitive tools. This is especially important when, as shown in Table 1, the range of MoCA scores was higher in both young and old intervention groups compared to their respective controls. It is possible that relatively small numbers of patients >45 years old could have skewed the results observed in that age group, and a larger sample in this group should be studied in the future. Also, a single dose is a limitation in this study.

Conclusion

Transcranial PBM has been gaining momentum over the last decade as a safe, noninvasive intervention in populations prone to neurocognitive decline. The results from this study provided additional evidence that PFC stimulation from a 1064-nm wavelength laser significantly enhances CCO photo-oxidation and cerebral oxygenation. Importantly, age was found to play a key role in the observed laser-induced metabolic and hemodynamic effects. Our findings support the use of TILS for cerebral oxygenation and alleviation of age-related decline in mitochondrial function, and for further research on its potential for neurocognitive enhancement in healthy and disease populations.

CRediT authorship contribution statement

Celeste L. Saucedo: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Project administration, Resources, Visualization, Writing – original draft. **Emily C. Courtois:** Investigation, Resources, Visualization, Project administration, Writing – review & editing. **Zachary S. Wade:** Investigation, Data curation, Project administration, Writing – review & editing. **Meghan N. Kelley:** Investigation, Resources, Data curation, Writing – review & editing. **Nusha Kheradbin:** Investigation, Resources, Data curation, Writing – review &

editing. **Douglas W. Barrett:** Methodology, Resources, Validation, Writing – review & editing. **F. Gonzalez-Lima:** Conceptualization, Methodology, Supervision, Project administration, Resources, Funding acquisition, Validation, Writing – review & editing.

Acknowledgements

This work was supported by generous grants from the Oskar Fischer Project and the Elhapa Foundation to Prof. Dr. F. Gonzalez-Lima. The authors thank Dr. Li-da Huang and Dr. Yingjun Zhao who were instrumental in the bbNIRS system setup and pre-processing of data; Patrick O'Connor for bbNIRS session training; Paola Bojorquez-Ramirez, Reagan Easley, Liliana Laboy-Pellicier, and Shane Mays for laser administration and collection of data, and Andrew Courtois for the bbNIRS setup figure. The authors also thank the participants for their time and contribution to science.

Published: 30 December 2024

The relationship between mitochondrial health, telomerase activity and longitudinal telomere attrition, considering the role of chronic stress

- Mauricio Guillen-Parra,
- Jue Lin,
- Aric A. Prather,
- Owen M. Wolkowitz,
- Martin Picard &
- Elissa S. Epel

Scientific Reports **volume 14**, Article number: 31589 (2024)

Abstract

Telomere attrition is a hallmark of biological aging, contributing to cellular replicative senescence. However, few studies have examined the determinants of telomere attrition in vivo in humans. Mitochondrial Health Index (MHI), a composite marker integrating mitochondrial energy-transformation capacity and content, may be one important mediator of telomere attrition, as it could impact telomerase activity, a direct regulator of telomere maintenance. In this observational longitudinal study, we examined in peripheral blood mononuclear cells (PBMCs), whether MHI predicted changes in telomerase activity over a 9-month period, thus impacting telomere maintenance over this same period of time. We secondarily examined the role of chronic stress, by comparing these relationships in mothers of children with an autism spectrum disorder (caregivers) vs. mothers of a neurotypical child (controls). Here we show that both chronic stress exposure and lower MHI independently predicted decreases in telomerase activity over the subsequent 9 months. Finally, changes in telomere length were directly related with changes in telomerase activity, and indirectly with MHI and chronic stress, as revealed by

a path analysis. These results highlight the potential role of chronic stress and MHI as drivers of telomere attrition in human PBMCs, through an impairment of both energy-transformation capacity and telomerase production.

Introduction

Rate of cell aging is complex and determined by many different measures of cellular function[1]. Telomere attrition is a well understood pathway leading to replicative senescence[2]. Telomeres are the nucleoproteins located at the ends of chromosomes, and are involved in maintaining genomic integrity[3]. Given that nuclear DNA cannot be fully replicated during mitosis (i.e. end replication problem), telomeres shorten with each round of cell division, unless acted upon by different telomere lengthening functions[4,5]. Telomeres generally shorten throughout life in humans and in cells critically shortened, telomeres promote cellular senescence or apoptosis[4,6]. Shorter telomeres are associated with higher risks of developing different diseases and mortality[7,8,9,10]. Thus, telomeres are considered not only as markers of chronological aging, but as markers reflecting biological aging and overall individual condition.

Rate of telomere attrition over years has rarely been studied longitudinally; however, it should reflect pace of replicative senescence, accumulation of telomere damage, for example through exposure to oxidative stress and hence, indicate immune system aging better than cross-sectional measures of telomere length[11]. Interestingly, telomeres can be maintained and even restored through different physiological pathways[12], such as the regulation of levels of telomerase enzymatic activity[2,13]. Telomerase contains a core RNA component (TERC) and reverse transcriptase (TERT), which together synthesize new telomeric repeats[2,13], plus some additional non-canonical functions that have been described[14,15,16]. As a result of the telomerase/telomere maintenance processes, there can be great between-individual differences in the rate of attrition/maintenance of telomeres. Thus, it is important to investigate what factors may be involved in the regulation of telomerase activity and, hence, telomere and cellular maintenance.

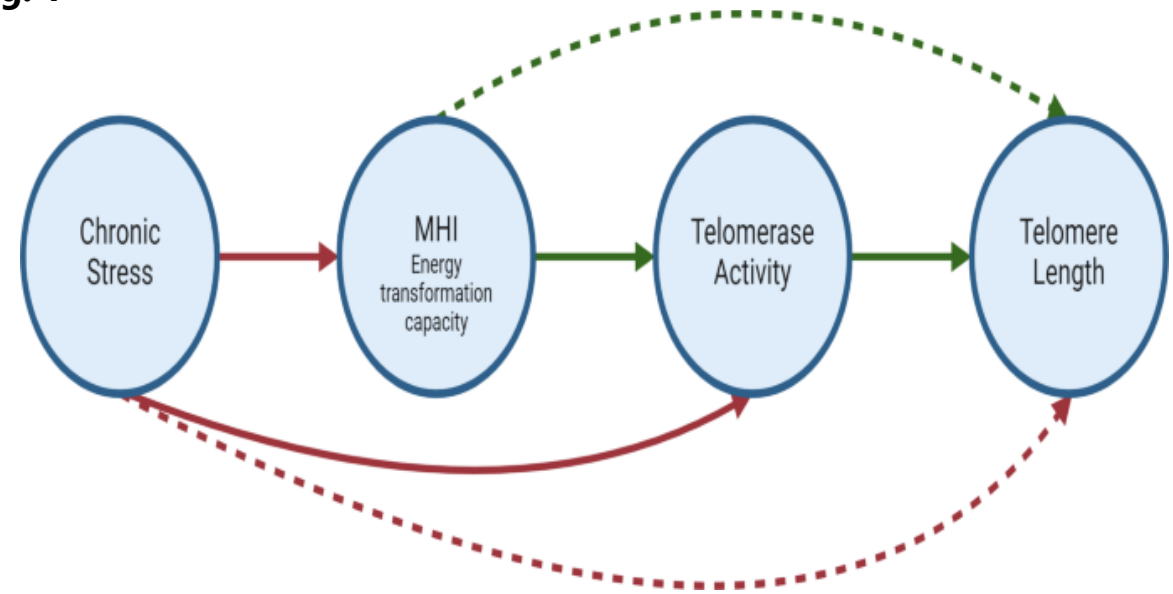
Telomerase activity in normal immune cells is negatively correlated with age in humans[17]. Further, telomerase activity in normal immune cells has been found to be negatively associated with certain lifestyle factors[18], such as smoking ([19], but see[20]), and to poor mental health, such as chronic stress[21,22]. Paradoxically, some studies have reported that high telomerase in peripheral immune cells, particularly in the context of shorter telomere length, is related to indices of chronic adversity and major depression[23,24]. Despite the importance of understanding telomerase regulation, no human studies we are aware of have examined potential upstream biological regulators of telomerase activity in in vivo settings. Furter, few studies in humans have measured telomerase activity, compared to telomere length. Telomerase activity estimation requires a highly unique assay methodology where both enzymatic activity preservation and RNA isolation are required, hence few studies have examined telomerase activity over time. Here, we examined, in vivo, whether a novel index of mitochondrial health has a role in the telomerase/telomere maintenance processes over time, as substantial evidence from in vitro studies suggest that mitochondria can regulate telomere maintenance, as reviewed below.

Mitochondria transform most of the energy required to power all basal cellular functions and the physiological stress response[25]. Several bidirectional biological pathways link mitochondria to telomere stability[26,27,28]. For example, telomere dysfunction can promote impaired mitochondrial respiration and biogenesis, probably through the activation of p53[26,29,30]. In turn, impaired mitochondrial biology can also lead to an increased telomere attrition[27], for example, through an increased production of reactive oxygen species (ROS), which can ultimately lead to a higher oxidative stress, and in consequence to a higher telomere attrition[31]. Furthermore, it has been reported that mitochondria are constantly interacting with both telomerase components[27], including TERT, which is proposed to bind to the mitochondrial DNA where it contributes to maintain its stability and function[32,33]. In turn, TERC has been found to be processed inside the mitochondria and then exported back to the cytosol, probably as a molecule giving information to the nucleus about the mitochondrial function[34,35]. Ultimately, it has been found in basic in vitro models that impaired mitochondrial respiration and impaired cellular bioenergetics can cause a faster telomere attrition[36], possibly through a

reduced capacity for telomerase production[37], as it affects production of other stress mediators[38].

Given the relationships described above, we infer both direct and indirect effects, relative to statistical testing, of chronic stress and mitochondrial-mediated low energetic capacity, over telomere maintenance, as shown in the model of Fig. 1. Importantly, these proposed relationships are only correlational, as no experimental manipulation was performed in any variable to properly test a causation effect.

Fig. 1



Direct and indirect pathways affecting telomere attrition. Model proposing the pathway regarding how chronic stress and Mitochondrial Health Index (MHI), through direct effects (solid lines) and indirect effects (dotted lines), could be regulating telomerase activity, and in consequence, telomere length. Chronic stress is linked to lower MHI[39] and telomerase activity[21] in previous studies. A downregulated MHI could dampen telomerase activity (to be tested here), presumably through an impaired energetic capacity[37]. In consequence, both chronic stress and MHI could be indirectly affecting telomere attrition rate, marked with dotted arrows. Green arrows indicate a positive relationship. Red arrows indicate a negative relationship. Figure created with BioRender.com.

In this study, we measured in mixed peripheral blood mononuclear cells (PBMCs) the mitochondrial health index (MHI), which integrates measures of mitochondrial respiratory capacity expressed relative to mitochondrial content, producing an index of energy transformation capacity[39]. We test relations between baseline MHI to the changes over time in telomerase enzymatic activity, and to changes in telomere length during a period of 9 months. We determined whether there are direct and indirect roles of MHI in predicting telomere attrition, such as proposed by the model in Fig. 1, by testing if it is a predictor of changes in the enzymatic activity of telomerase and telomere length over the 9-month period. We predicted that individuals with a higher MHI at baseline will evidence a relatively smaller decrease in their telomerase activity over time, compared to individuals with lower baseline MHI. We hypothesized that both MHI and changes in telomerase activity will be associated with changes in telomere length, where individuals with a higher MHI and an increased telomerase activity will have better telomere maintenance over time.

Lastly, we tested if these relationships are moderated by chronic psychological stress (Fig. 1), by comparing mothers of a child with an autism spectrum disorder (i.e. stressed caregivers) with mothers of a neurotypical child (i.e. lower stress control). In this sample, we have previously found that chronic psychological stress is related to worse mitochondrial health[39]. Thus, chronically-stressed mothers with a lower MHI at baseline may have a more rapid decrease in telomerase activity and telomere shortening over a 9-month period.

Results

Demographic and baseline biological indices by chronic stress status

The sample of 85 mothers, across both chronic stress status (i.e. caregiver or control), were 43.46 ± 4.96 years old on average, and age did not differ between groups (Table 1). The sample had an average BMI of 25.30 ± 5.00, being also similar between groups (Table 1). Further, BMI did not change on average during the 9-month period (paired t test, $t = -0.38$, $p = 0.71$).

Table 1 Differences between mothers of children with an autism spectrum disorder (caregivers, *n* = 45) and mothers of a neurotypical child (control, *n* = 40) in their demographic characteristics and biological indices at baseline.

The Mitochondrial Health Index (MHI) was different between the two chronic stress groups (Table 1), as previously reported[39], where caregiver mothers had a lower MHI compared to control mothers (Table 1). Finally, both baseline telomerase activity and telomere length did not differ between high and low chronic stress groups (Table 1).

Biomarkers by age and BMI

MHI was not related with individuals' age or BMI (Table 2). Further, telomerase activity at baseline was also not associated with age or BMI (Table 2). Finally, telomere length at baseline was predicted by the individuals' age (Table 2), as older individuals had shorter telomeres ($\beta \pm SE = -0.36 \pm 0.08$, $p < 0.01$), but it was not related with BMI (Table 2).

Table 2 Results from linear regression models evaluating the effect of the individuals' age and BMI over the different biological indices at baseline, and over the change in telomerase activity and telomere length after a 9-month period.

The change in telomerase activity and the change in telomere length over the 9-month period were not predicted by the individuals' age or BMI (Table 2).

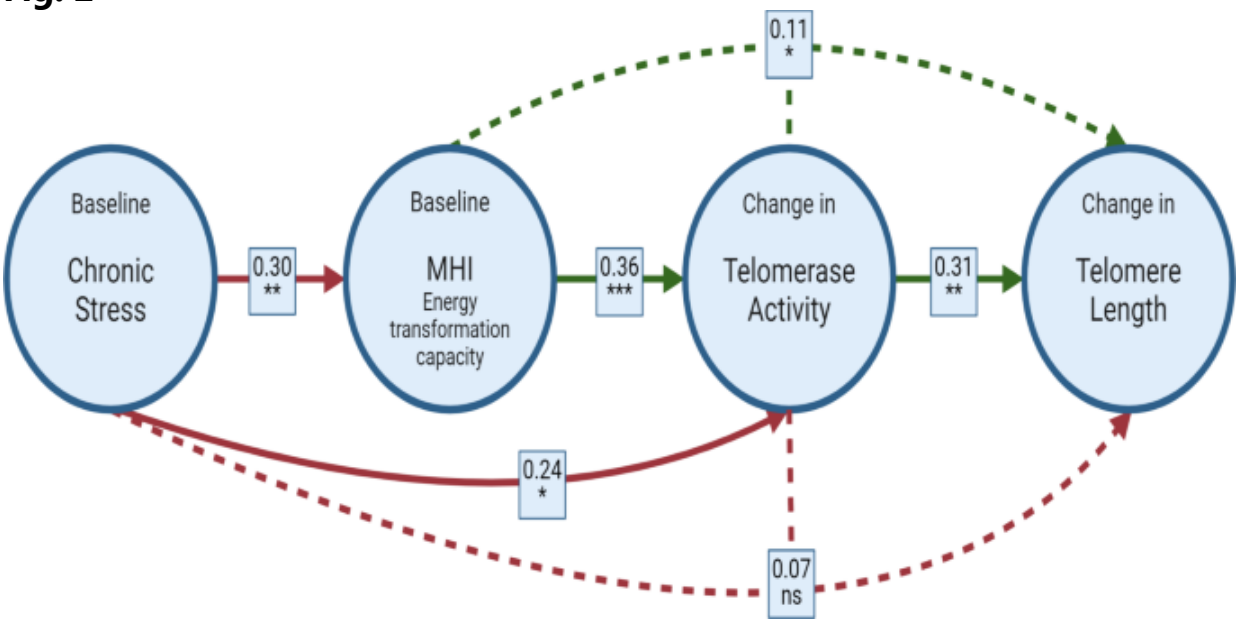
Correlations between biological indices at baseline

Telomerase activity at baseline was not significantly related to MHI ($F_{1,79} = 2.21$, $p = 0.14$) or to chronic stress status ($F_{1,79} = 2.39$, $p = 0.12$). Further, telomere length at baseline was also not related to MHI ($F_{1,74} = 0.31$, $p = 0.58$), telomerase activity ($F_{1,74} = 0.02$, $p = 0.90$) or chronic stress group ($F_{1,74} = 0.04$, $p = 0.85$).

Chronic stress, MHI, and changes in telomerase activity and telomere length over time

The path analysis indicated several direct and indirect effects between our biological indices (Fig. 2).

Fig. 2

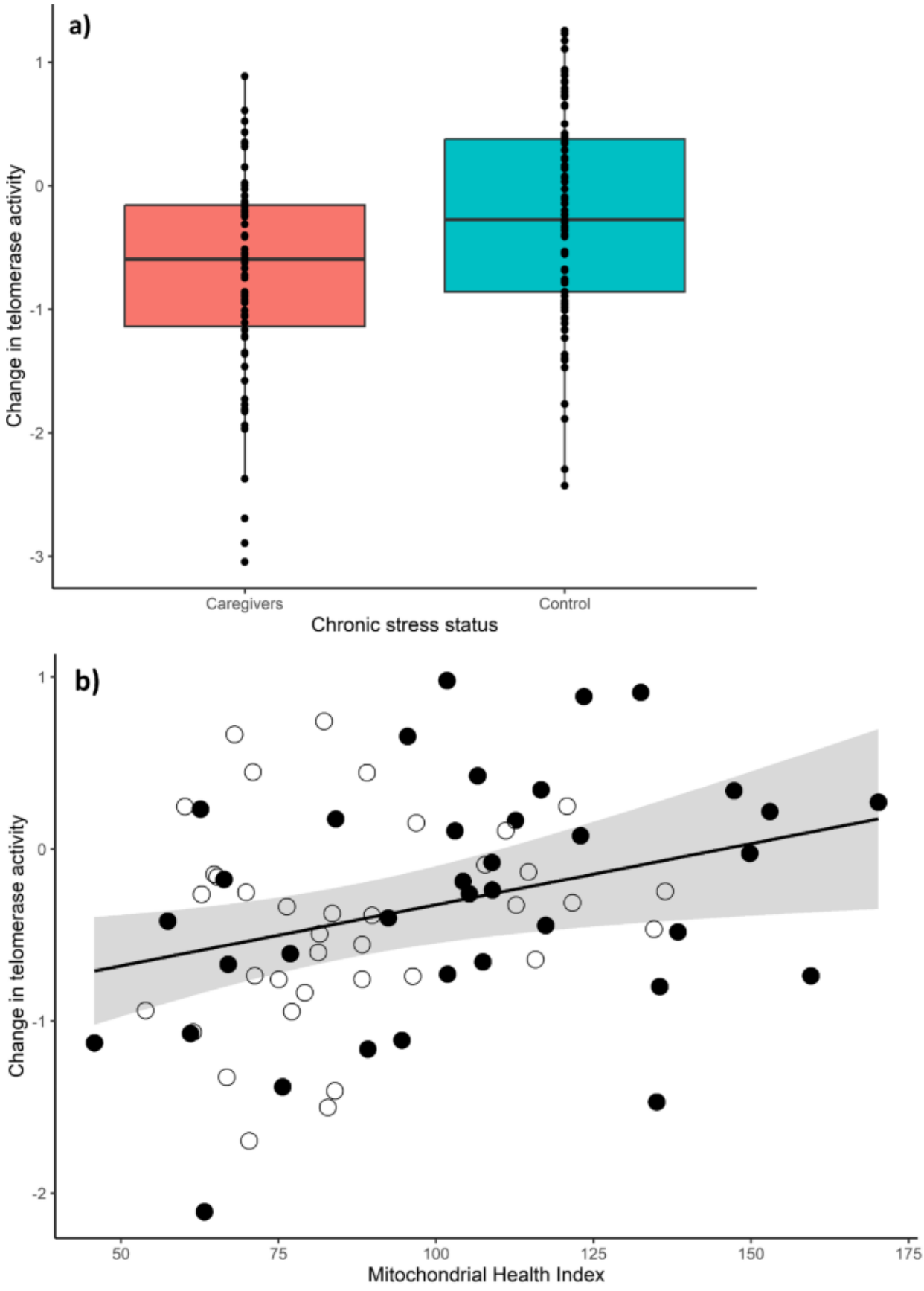


Path model and coefficients for the hypothesized relationships in Fig. 1 leading to telomere attrition. Path analysis testing the direct and indirect effects between chronic stress status (caregiver vs. control mothers), Mitochondrial Health Index (MHI) at baseline, and changes in telomerase activity and telomere length over a 9-month period. Solid arrows indicate direct effects between terms. Dotted arrows indicate indirect effects of chronic stress status and MHI over the change in telomere length, through their effects on another intermediary variable (telomerase activity). In this figure, the numbers indicate the standardized estimated effect of each relationship, and if the effect is significant is noted with asterisks (ns: not significant). Red arrows reflect negative effects. Green arrows reflect positive effects. Figure created with BioRender.com.

Regarding the direct effects, although only correlational, first we see that chronic stress status is associated with MHI, as reported above ($Z = 2.65$, $p < 0.01$). Then, the change in telomerase activity in the 9-month period is negatively related with the chronic stress status ($Z = 2.24$, $p = 0.02$) and positively with the MHI ($Z = 3.36$, $p < 0.01$). Caregiver mothers show a greater decrease in their telomerase activity (-0.73 ± 0.83), than control mothers (-0.25 ± 0.84; Fig. 3a). As predicted, higher baseline values of MHI predict a longitudinal maintenance in telomerase activity, while lower baseline MHI values are associated with a decrease in telomerase activity (Fig. 3b), and

this association did not differ by chronic stress group (MHI * Chronic stress $F_{1,68} = 0.24$, $p = 0.63$).

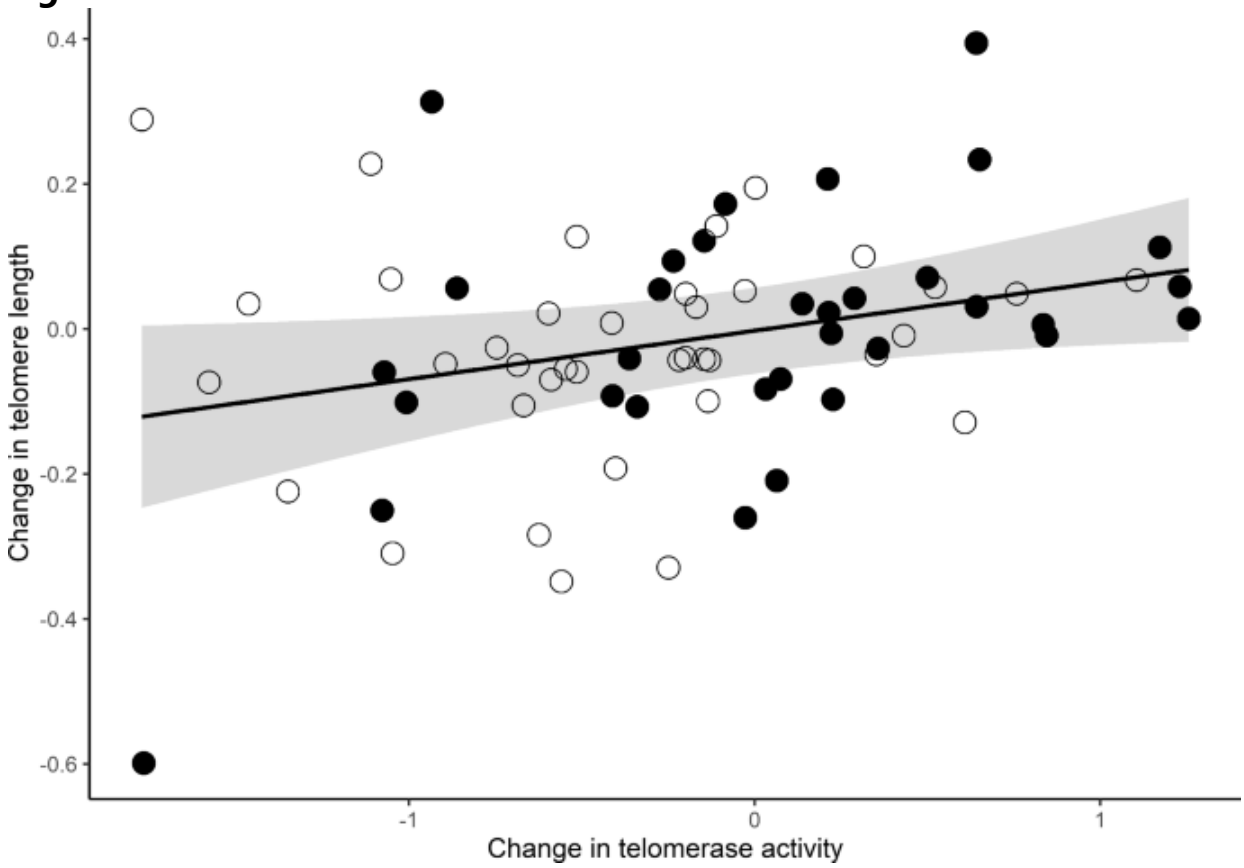
Fig. 3


a)
Change in telomerase activity
1
0
-1
-2
-3
Caregivers
Control
Chronic stress status
b)
Change in telomerase activity
1
0
-1
-2
50
75
100
125
150
175
Mitochondrial Health Index

Effects of chronic stress status and Mitochondrial Health Index over the change in telomerase activity after a 9-month period. Changes in (ln) telomerase activity (units/10,000 cells) over a 9-month period explained by the (**a**) chronic stress status (caregivers vs. control mothers), and (**b**) Mitochondrial Health Index (MHI), where open circles are stressed caregiver mothers and closed circles are control mothers, shaded area reflects the confidence interval (95%) for the estimated slope. Positive values indicate an increase in telomerase activity, and negative values indicate a decrease. Model's $r^2 = 0.24$, $p < 0.001$.

The change in telomere length over the 9-month period was not directly predicted by the chronic stress status ($Z = -0.56$, $p = 0.57$) nor by MHI at baseline ($Z = -0.08$, $p = 0.94$). As expected, the change in telomere length was directly related to the change in telomerase activity ($Z = 2.38$, $p = 0.02$). An increase in telomerase activity is associated with a better maintenance of telomere length (Fig. 4). This relationship did not significantly differ between chronic stress groups, although there were marginal differences (Telomerase change * Chronic stress $F_{1,64} = 3.45$, $p = 0.07$). Control mothers showed a positive relationship between the change in telomerase activity and the change in telomere length ($\beta \pm SE = 0.39 \pm 0.14$, $p < 0.01$), as shown in Fig. 4. However, in the stressed caregivers group the slope of the relationship is more flattened ($\beta \pm SE = 0.03 \pm 0.23$, $p = 0.90$).

Fig. 4



Relationship between the change in telomerase activity and the change in telomere length after a 9-month period. Changes in (ln) telomere length (T/S ratio) over a 9-month period explained by the changes in (ln) telomerase activity (units/10,000 cells) over the same period of time. Positive values indicate an increase in telomerase activity and telomere length, and negative values indicate a decrease. Open circles are stressed caregiver mothers, closed circles are control mothers. Shaded area reflects the confidence interval (95%) for the estimated slope. Model's $r^2 = 0.10$, $p = 0.05$.

Lastly, the quantification of the indirect effects indicated that, chronic stress status, through its effects on telomerase activity, does not have a statistically significant indirect effect on the change in telomere length ($Z = 1.63$, $p = 0.10$, Fig. 2), although there is the expected trend towards it. Finally, MHI has an indirect effect on the change in telomere length over time, through its effects on the change in telomerase activity ($Z = 1.94$, $p = 0.05$, Fig. 2).

Discussion

In this study, we found that changes in telomerase activity over time, in human PBMCs, appear partly regulated by mitochondrial health (Mitochondrial Health Index, MHI). Individuals with higher baseline MHI, indicating greater energy transformation capacity, maintained higher active telomerase activity during a 9-month period, compared to individuals with lower MHI, who on average experienced drops in their telomerase activity. Further, the high chronic stress group (caregiving mothers) showed both reduced MHI and a faster decrease in their telomerase activity. Hence, a chronic stressor may amplify the impact of lower energetic capacity on maintaining telomerase activity. Ultimately, having lower MHI, associated with reduced telomerase activity, may speed the rate of telomere attrition, a marker of cellular senescence that has been associated with higher disease and mortality risks, although disease and mortality were not independently assessed in this study. Overall, the predicted relationships of both direct and indirect statistical effects of the model shown in Fig. 1 were supported. These are the first findings we are aware of in humans tracking changes in telomerase activity and telomere length over time, and highlight a novel demonstration of new pathways through which chronic stress and mitochondria may affect telomere dynamics in vivo, described further below.

Potential mechanisms of mitochondrial health impact on telomerase

Our results suggest that our index of mitochondrial health (MHI) serves as a buffer or positive prognostic factor, as it predicts, in PBMCs, better maintenance of telomerase activity and hence telomere length during a 9-month period. The MHI is a marker reflecting energy transformation capacity per unit of mitochondria content (see Methods), which has been found to be superior in describing mitochondrial "quality" than measures of respiratory capacity or content alone[39]. A higher mitochondrial functional capacity could impact telomerase activity through a higher energy availability. Cells with limited mitochondrial energy transformation capacity (low MHI) may presumably experience greater energetic stress under certain conditions such as immune activation or oxidative stress. Because each cell has a limited energy budget that constrains cellular activities, energy-demanding stress responses can trigger the diversion of energy towards stress processes, and

away from longevity-promoting growth, maintenance, and repair (GMR) processes including somatic maintenance and telomerase activity[37]. Such maladaptive energy tradeoff may preferentially affect cells with lower MHI, thus conferring the apparent vulnerability to telomere attrition observed in our study.

Alternatively, higher MHI could also reduce or stop the exportation of telomerase components TERT and TERC from the nucleus, as they are imported into the mitochondria especially during times of high ROS production and oxidative stress, in order to buffer against mitochondrial stress[32,33]. This could allow for a higher telomerase activity in the nucleus, which can ultimately impact on telomere maintenance. However, telomerase components' non-canonical functions in the nucleus and the mitochondria are not fully understood, and may be independent of one another and from the overall enzymatic activity of telomerase[15,35]. Our results raise the possibility that mitochondria may regulate telomerase activity dynamics over time, but whether this happens due to energetic trade-offs or through another pathway, such as TERT and TERC importation dynamics, remains to be tested in the future. Finally, it could be possible that the apparent regulation of telomerase activity through MHI may also affect the different non-canonical functions of the telomerase[14,15,16], however this was not assessed in this study.

What does stress have to do with it?

1. a)

   Chronic stress affects mitochondria activity.

We previously reported that mothers caregiving for a child with an autism spectrum disorder have a lower MHI[39]. MHI can be affected by the emotions and mood of the individuals, even from the hours and days before sampling[39]. Supporting this notion, it has been reported that having a longitudinal higher well-being, evaluated through different psychosocial factors, is related to a higher abundance of mitochondrial OxPhos in postmortem individuals' brain[40]. Furthermore, mitochondrial respiration and content in PBMCs have been found to be altered in women that have experienced early life stress,

reflecting allostatic load[41]. Thus, living with chronic social stress could chronically impair mitochondrial functioning. In male rodents, experimental manipulations have demonstrated that chronic stress can have negative effects on the mitochondrial capacity of individuals[42]. Finally, in humans, at the cellular level, it has been reported that chronic stress can affect both mitochondrial functioning and content, leading to a cellular hypermetabolic state, associated with an increased cellular energy expenditure[36].

1. b)

Chronic stress suppresses telomerase activity.

Chronic stress plays an increasingly recognized important role in cellular aging[43,44] Individuals experiencing a chronically stressful life situation, such as caregiving stress, have been found to have shorter telomeres[45,46]. Furthermore, basal telomerase activity is another trait possibly affected by chronic stress[47,48,49], although only a handful of studies have evaluated this relationship in humans. In women, caregivers of either children with autism or dementia patients were associated with lower telomerase activity than control non-stressed women[21,50]. However, opposite results have also been reported, as men and women caregiving for Alzheimer patients show higher telomerase activity compared to control people[51]. Further, in a sample of healthy women, individuals reporting higher perceived chronic stress showed lower telomerase activity[52]. Finally, in older men, high telomerase activity is reported in those having experienced a reduced social support, lower optimism and higher hostility and early life adversity[53]. Regarding mental disorders and their relationship with telomerase activity, although mixed results have been reported[47], individuals with major depressive disorder seem to consistently show increased peripheral telomerase activity, particularly in men[24,47,54,55]. The present study extends the existing findings by showing that longitudinally, chronic stress, at least in the studied population, differs than depression, and leads to dampened telomerase activity over time, underlying a faster rate of cellular aging.

In this study, we found that the effects of chronic stress on telomerase activity could be partly mediated by the lower mitochondrial health, and subsequent

energetic stress, that is found in mothers caregiving for a child with an autism spectrum disorder. Additionally, chronic stress was directly related with the change in telomerase activity over time, independently of their relationship with MHI. Chronic stress can affect telomerase activity through different non-exclusive pathways. For example, individuals under a chronic stress condition are reported to have an altered cortisol secretion[56,57] and an increased oxidative stress[58]. In turn, chronic oxidative stress is reported to reduce telomerase activity[59], while glucocorticoids are found to regulate telomerase activity in different directions[60,61]. Thus, the direct relationship of chronic stress over telomerase activity dynamics found here, is likely mediated by other pathways not considered in this paper.

Mitochondrial health indirectly predicts telomere length attrition

Telomere length and its rate of attrition are markers associated with cellular senescence, reflecting biological aging, since they can predict the individuals' risk of developing different diseases and all-cause mortality[7,8,9,10]. Hence, it is of great relevance to study how telomerase, a main precursor of telomere maintenance and lengthening, can be regulated over time, as this could directly affect telomere attrition. In this study we found that telomere dynamics can be indirectly altered by both chronic stress and mitochondrial health, as both of them are related to telomerase activity dynamics. In line with our results, in a sample of healthy adults it was found that childhood adversity and lifetime psychopathology were related to an increased mitochondrial DNA copy number and to shorter telomeres[62]. This supports the notion that chronic stress may impair both mitochondrial biology and ultimately telomere maintenance, probably through its effects on telomerase activity, as our results suggest. Interestingly, chronic stress is generally accompanied by a greater perceived stress, while mitochondrial health can be affected by the emotions and mood experienced[39]. Therefore, it would be important to evaluate if different interventions, aiming to reduce stress, could impact on individuals' mitochondrial biology and telomerase activity, as these seem to improve telomere maintenance[63], yet this idea was not evaluated in this paper.

Overall, our results in PBMC's align with recent experimental findings linking impaired mitochondrial respiratory capacity to accelerated telomere shortening rates in cultured human cells[64]. In primary human fibroblasts aged over a 9-month period in vitro, inhibiting mitochondrial respiration accelerated telomere shortening rate by a 7.69-fold[65]. This finding was replicated in cells of individuals with a genetically-defined mitochondrial defect (SURF1 mutation), in which telomere attrition rate was on average 1.62-fold faster than in control cells with normally functioning mitochondria[65]. Moreover, on both models, cells with low mitochondrial health also exhibited accelerated epigenetic aging, based on epigenetic clocks trained to predict age in human tissues[65]. This suggest that the accelerated telomere shortening induced by mitochondrial defects indeed reflects accelerated biological aging, supporting the results of the present study.

Limitations. Finally, it is worth noting that our measures of mitochondrial health, telomerase activity and telomere length were all calculated in peripheral blood mononuclear cells (PBMCs). There is evidence indicating differences in telomerase activity and telomere length between the different immune cell types[66], where B cells seem to have higher telomerase activity and longer telomeres than T cells[67]. Additionally, mitochondrial function can also differ between B and T cells, and in T cells, activated and inactivated cells may differ in both mitochondrial activity and content[68]. Thus, our results showed here could be partly influenced by differences in cell composition between individuals, and within individuals after the 9-month period. Further, MHI was related with the time the samples spent in the freezer (see Methods). However, we cannot fully rule out the potential confound of freezer time statistically, as it is also confounded with the chronic stress, since samples from caregivers were mostly collected before samples from control mothers. Differences in storage time could indeed affect levels of mitochondrial enzymatic activity. However, when running separate analyses for caregivers and controls, MHI is still related to psychological distress, even after controlling for storage time differences between individuals[39], suggesting that differences in MHI are indeed linked to the chronic stress of the individuals[39]. Further studies are needed to replicate the chronic stress/mitochondria effect. Another limitation is that we only measured MHI at one timepoint. We therefore do not know how stable it was over time for individuals in our study.

We know from work in progress that PBMCs MHI has high stability within the day, and we observe changes on average from 4 to 6% from morning to evening (69 in progress). Further, in a study of one subject sampled over time, MHI showed some stability but also changed over weeks70. Despite the possible change over time in MHI within individuals, which would tend to decrease/underestimate true effect sizes, we nevertheless found that the individuals' baseline MHI still predicted their changes in telomerase activity and telomere length. Lastly, telomerase activity in brain may be regulated differently from that in peripheral blood cells71, so the present findings should not be uncritically extrapolated beyond PBMCs.

Conclusion

In summary, our data in PBMCs from healthy midlife women show that chronic stress and low mitochondrial health are associated with a more pronounced reduction of telomerase activity over a 9-month period. These effects consequently contribute to an accelerated rate of telomere attrition, shown by the association between a decrease in telomerase activity and accelerated telomere attrition. These results propose an alternative pathway implicating reduced mitochondrial energy transformation capacity as a harbinger of telomere attrition, a process exacerbated in presence of chronic stress.

Methods

Data for this secondary analysis study were derived from a larger longitudinal study called "Stress, Aging, and Emotions (SAGE)", which focused mainly on the effects of caregiving stress on cellular aging, and was composed by a total of 183 mothers (92 stressed caregiver mothers and 91 control mothers). All individuals were recruited in the San Francisco Bay Area, either by schools, mailing, social media, or directly through the University of California San Francisco Autism Clinic. Participants were eligible only if they were non-smokers and had an age between 20 and 50 years old, having at least one child between the ages of 2 and 16 years. For the caregiver mothers, at the beginning of the study the mean duration of years of caregiving was 5.1 years (range = 1.1–13.9). All study participants reported being premenopausal and in good health with no major medical conditions. During the time of the study, only two participants, both in the stressed caregivers group, met the

diagnostic criteria for depression, and no participants were taking any hormonal birth control medication. This study was approved by the Institutional Review Board at the University of California, San Francisco, and all methods were performed in accordance with the relevant guidelines and regulations. Finally, written informed consent was obtained for each study participant.

For a subgroup of 85 participants (caregivers = 45, control = 40) we had enough preserved PBMCs to quantify the mitochondrial health index (MHI), along with the telomere length (n = 81) and telomerase activity (n = 85; see below for details). Blood samples were collected at baseline, and again 9 months later to measure changes in both telomerase activity (n = 75) and telomere length (n= 75). We were unable to measure changes in MHI at the second sampling point. There was no intervention between the time points. Both groups in this subsample did not differ in any sociodemographic or health factors [see[39] for full details].

Mitochondrial Health Index (MHI)

To obtain an index of mitochondrial health (MHI), we followed a protocol previously reported[39]. Briefly, to calculate the MHI we calculated 4 different parameters related to both mitochondrial function and content. As markers of mitochondrial function, we quantified the activity of two enzymes related to two complexes of the mitochondrial respiratory chain: succinate dehydrogenase (SDH) a marker of complex II activity, and cytochrome c oxidase (COX) an activity marker of complex IV. And as markers of mitochondrial content, we quantified the enzymatic activity of citrate synthase (CS) and the number of mitochondrial DNA copy number per cell (mtDNAcn). All enzymatic activities were quantified spectrophotometrically, and mtDNAcn was calculated through quantitative real-time PCR [see[39] for details]. All four parameters were mean-centered. Then, SDH and COX were added and included as a numerator, and CS and mtDNAcn were also added and included as a denominator. As a result, our measure of MHI reflects respiratory chain capacity per unit of mitochondrial content[39].

Our estimate of MHI was found to be affected by the time the samples spent in the freezer. However, time spent at the freezer also differed between both chronic stress groups. Thus, chronic stress status and freezer time are confounded variables so it was impossible for us to covary for freezer time in the statistical analyses without completely removing the chronic stress effect[39].

Telomere length and telomerase activity

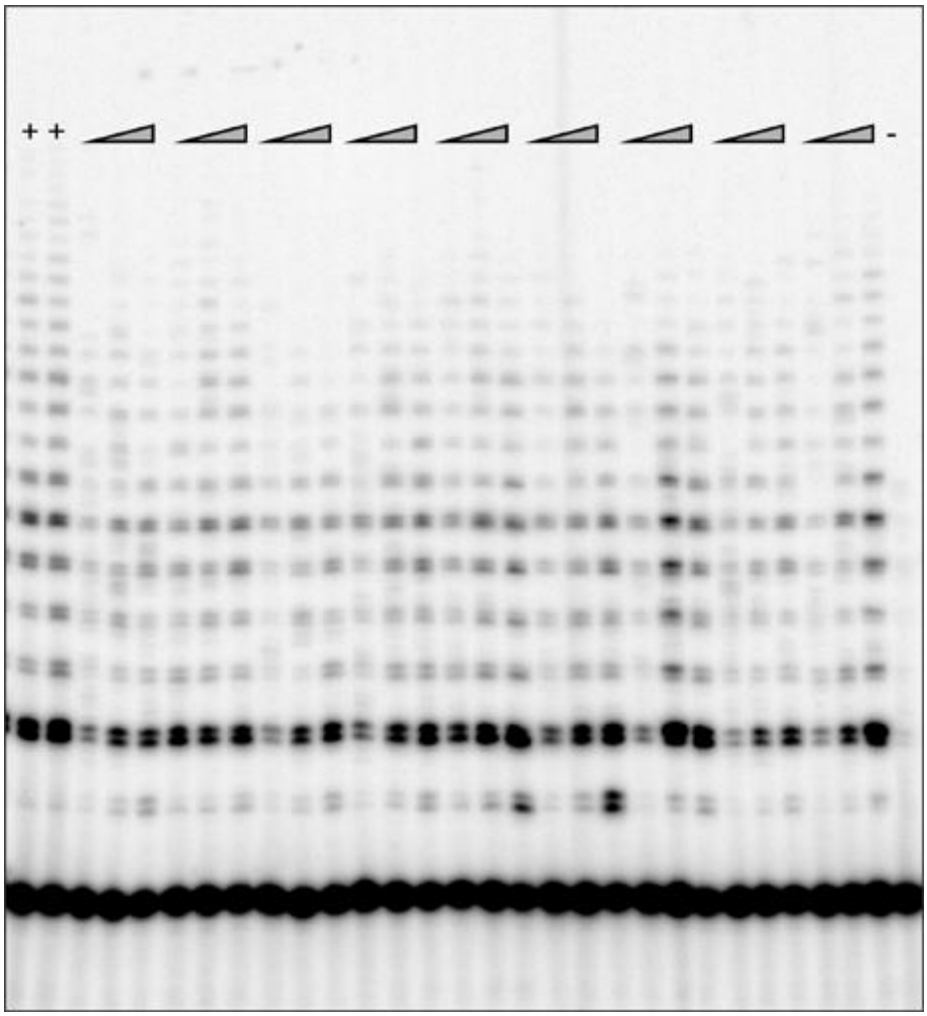
Telomere length was quantified in peripheral blood mononuclear cells (PBMCs). PBMCs were isolated from whole blood samples by Ficoll Hypaque density gradient centrifugation within 6 h from blood collection, and then were conserved at -80 °C until laboratory analyses. DNA was extracted from PBMCs and then purified using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany, Cat. Number 51104). To estimate telomere length, we used quantitative real time PCR, following a protocol previously reported[67], which was adapted from the original method published by Cawthon[72], which ultimately gives us a measure of telomere length controlling for a single-copy nuclear gene (T/S ratio). Eight samples were included in each plate to control for interassay variability. Each sample was measured in duplicates, and if intraassay variability was higher than 7%, a third measure was performed.

Due to logistical reasons, we analyzed all samples in two different batches separated in time. To control for variance between batches, we used the same 8 samples used to measure interassay variability in both batches. We then compared the T/S ratio of these samples from both batches, and calculated a correction factor used only for the samples in the second batch, in order to make them comparable to the samples from the first batch. The adjusted value was calculated as follows: (Second batch value – 0.04472) / 0.8676.

Telomerase activity was also quantified in PBMCs. Telomerase activity in PBMCs was quantified using a commercial kit (TRAPeze Telomerase Detection Kit, Millipore), where gel-TRAP assays were performed by the Telomerase Repeat Amplification Protocol (TRAP), as previously reported[67]. Briefly, after purification of the PBMCs, 5×10^5–1×10^6 cells per sample were pelleted and lysed with 1XCHAPS buffer. Extracts corresponding to 5000 cells/μL were

analyzed in batches, on an 8% polyacrylamide-8 M urea sequencing gel[67]. Then, the gel was exposed to a phosphorimager plate overnight and scanned on a Typhoon 8600 Imager (GEHealthcare, Piscataway, NJ), including both positive and negative controls along with the focal samples (Fig. 5). As a standard and positive control of telomerase activity we used the 293T cancer cell line, where estimates were expressed as equivalent of the number of 293T cells[67]. Finally, telomerase activity was quantified using the software ImageQuant 5.2 (GE Healthcare, Piscataway, NJ), where, after subtracting the background, signals from the product ladders on the gels were normalized and added against the signal from the internal control band for the same lane to get the product/internal control value, doing the same also for the negative control lane[67].

Fig. 5



Example of a gel used for the telomerase activity estimation. In this figure, + represents the positive control, where 10 cells of 293T cancer cell line were used, - represents the negative control, and each triangle represents the increasing concentrations in the number of cells used for each focal individual (2500, 5000 and 10000 cells).

For each telomerase activity assay reaction, the product/internal value for the sample was subtracted by the product/internal value for negative control and divided by the product/internal control value - negative control product/internal value from ten 293T cells and then multiplied by 10 to obtain the final telomerase activity units, defined as 1 unit = the amount of product from one 293T cell/10,000 immune cells[67].

Telomerase activity and telomere length (T/S ratio) were both natural log-transformed in order to meet linear models' assumptions.

Statistical analytic plan

First, we tested if our two groups of chronic stress status (caregivers and controls) differed in two demographic variables: age and BMI, and in the different biological indices at baseline: MHI, telomerase activity and telomere length. This was done by doing independent t tests.

Then, we tested if our different biological indices at baseline were affected by the individuals' age or BMI, by doing independent linear regression models where the biological indices were the response variables, and age and BMI were the explanatory terms. Later, we quantified the change in telomere length and telomerase activity over the 9-month period through the difference between both sampling points (Second measure – First measure), where negative values would indicate a decrease in either telomerase activity or telomere length, and in turn, positive values would indicate an increase. We also tested if the change in telomerase activity and telomere length were affected by the individuals' age or BMI following the same procedure described above.

We then also explored for relationships among our biological indices at baseline. First, we tested if telomerase activity was related to MHI, and if telomere length was related to MHI and telomerase activity, by doing independent linear regression models, where chronic stress status and individuals' age were included as covariates.

Finally, to test the direct and indirect effects that chronic stress status and MHI can have over the changes in telomerase activity and telomere length over time, as predicted in the model in Fig. 1, we performed a path analysis using the *lavaan*package version 0.6–17 in R[73]. Age was only associated with telomere length at baseline. Since age and BMI were not associated with MHI, nor with the change in both telomerase activity and telomere length over time (Table 2), they were excluded from the path analysis described below. The path analysis consisted of 3 different linear regression models testing direct effects.

The first model included the change in telomere length as the response variable, and MHI, chronic stress status and change in telomerase activity as the explanatory variables. The second model included the change in telomerase activity as the response variable, and MHI and chronic stress status as the independent terms. Finally, the third model included MHI as the response variable, and chronic stress status as the explanatory term. For exploratory purposes, we tested if the effects of MHI on the change in telomerase activity, and the effects of the change in telomerase activity on telomere length change, were dependent on the chronic stress status, by testing an interaction between these terms. However, since any of the interactions resulted significant (see Results), they were dropped from final models to remain with interpretable estimates of main effects[74]. Lastly, by using the default "Delta method" option[73], we calculated indirect effects of: chronic stress status over changes in telomere length (through changes in telomerase activity), and MHI over changes in telomere length (through changes in telomerase activity).

In all models, we confirmed that model assumptions were met by graphical visualization of the residuals. Data were analyzed using R software version 4.1.2 (R Core Team 2021). Mean and standard deviations (SD) are shown

throughout the text, and effect sizes (i.e. r^2) of significant models are shown in the figure legends. Statistical significance was set at a p value ≤ 0.05.

Published: 14 March 2023

Cellular rejuvenation: molecular mechanisms and potential therapeutic interventions for diseases

- Shuaifei Ji,
- Mingchen Xiong,
- Huating Chen,
- Yiqiong Liu,
- Laixian Zhou,
- Yiyue Hong,
- Mengyang Wang,
- Chunming Wang,
- Xiaobing Fu &
- Xiaoyan Sun

Signal Transduction and Targeted Therapy **volume 8**,
Article number: 116 (2023)

Abstract

The ageing process is a systemic decline from cellular dysfunction to organ degeneration, with more predisposition to deteriorated disorders. Rejuvenation refers to giving aged cells or organisms more youthful characteristics through various techniques, such as cellular reprogramming and epigenetic regulation. The great leaps in cellular rejuvenation prove that ageing is not a one-way street, and many rejuvenative interventions have emerged to delay and even reverse the ageing process. Defining the mechanism by which roadblocks and signaling inputs influence complex ageing programs is essential for understanding and developing rejuvenative strategies. Here, we discuss the intrinsic and extrinsic factors that counteract cell rejuvenation, and the targeted cells and core mechanisms involved in this

process. Then, we critically summarize the latest advances in state-of-art strategies of cellular rejuvenation. Various rejuvenation methods also provide insights for treating specific ageing-related diseases, including cellular reprogramming, the removal of senescence cells (SCs) and suppression of senescence-associated secretory phenotype (SASP), metabolic manipulation, stem cells-associated therapy, dietary restriction, immune rejuvenation and heterochronic transplantation, etc. The potential applications of rejuvenation therapy also extend to cancer treatment. Finally, we analyze in detail the therapeutic opportunities and challenges of rejuvenation technology. Deciphering rejuvenation interventions will provide further insights into anti-ageing and ageing-related disease treatment in clinical settings.

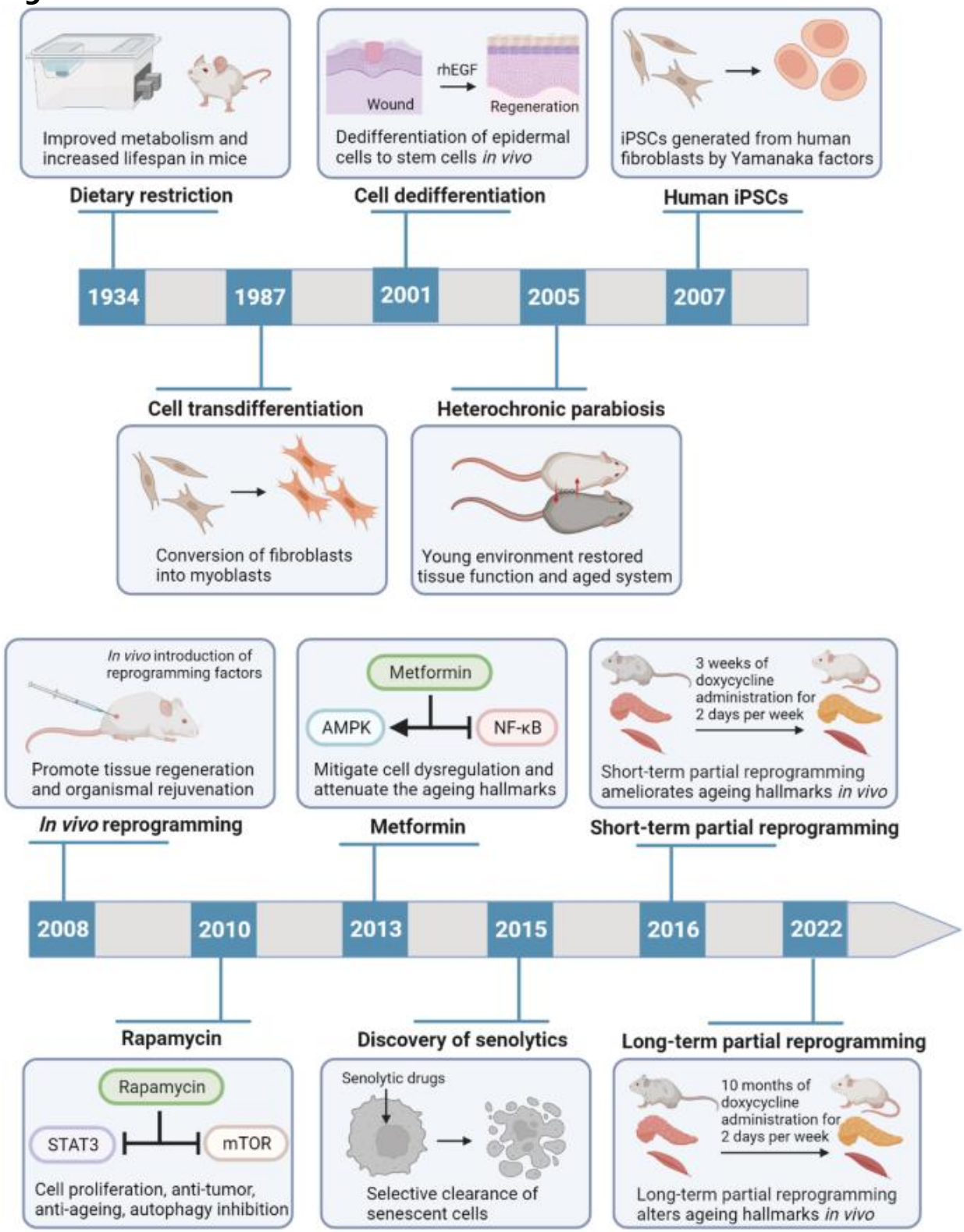
Mechanisms, pathways and strategies for rejuvenation through epigenetic reprogramming

Article 15 December 2023

Introduction

Ageing is a dynamic and time-varying process, typically manifested by cell damage accumulation, degeneration of tissue and organ structure and function, and increased susceptibility to diseases.[1] As the risk factor for human mortality, ageing is closely associated with many chronic diseases, like diabetes, Alzheimer's disease (AD), chronic kidney diseases (CKD), cardiovascular diseases (CVD), and cancer.[2] Therefore, enhancing the knowledge of ageing and the development of rejuvenation interventions are priority targets in biomedical research. Dietary restriction (DR) was found to prolong lifespan in mice and rats in 1934, and there are currently many emerging rejuvenation treatments to enhance health and lengthen lifespan, such as genetic, pharmacological, dietary, and lifestyle modifying approaches (Fig. 1).[3] However, these breakthroughs were obtained in short-lived organisms from yeast model to mice model.[4] Considering the complex anti-ageing mechanisms, it still takes a long translational phase to implement rejuvenation interventions into clinical applications.

Fig. 1



The milestone events for cellular rejuvenation research advances. Starting with the 1934 discovery of the influence of dietary restriction on lifespan extension,

important findings on the subject of cellular rejuvenation are emphasized. More recently, the senolytics development and reprogramming technology have been widely applied for cellular rejuvenation. rhEGF recombinant human epidermal growth factor, iPSCs induced pluripotent stem cells, AMPK 5′-AMP-activated protein kinase, NF-κB nuclear factor-κB, STAT3 signal transducer and activator of transcription 3, mTOR mammalian target of rapamycin. Created with BioRender.com

Rejuvenation usually refers to giving aged cells or organisms more “youthful” characteristics through various techniques, such as cellular reprogramming and epigenetic regulation.[5] Especially, the technique using induced pluripotent stem cells (iPSCs) in vitro via Yamanaka transcription factors is becoming increasingly proficient in the methodology and applications.[6] Different organisms share certain molecular and cellular characteristics that are indicative of ageing, such as cellular senescence, epigenetic changes, telomere attrition, genomic instability, stem cell exhaustion, deregulated nutrient sensing, loss of proteostasis, mitochondrial dysfunction, and altered intercellular communication.[7] Targeting these hallmarks of ageing is thus a growingly crucial field of research for the development of innovative cellular rejuvenation strategies. Nevertheless, these interventions have been lacking core criteria of rejuvenation in anti-ageing development and human applications.[8] It is necessary to accurately define rejuvenation and characterize the therapeutic effects systemically. To understand whether the ageing can be rejuvenated, it also needs to uncover the common or distinct mechanisms underlying rejuvenation in different cell types that build each organ.[9] In addition, a complete framework that describes various rejuvenation strategies should be established, contributing to cellular rejuvenation applications in human diseases. Herein, we provide a systematic and comprehensive discussion of cellular rejuvenation mechanisms and therapeutic interventions. An in-depth understanding of the pivotal roles will provide further insights into cellular rejuvenation in human disease treatment.

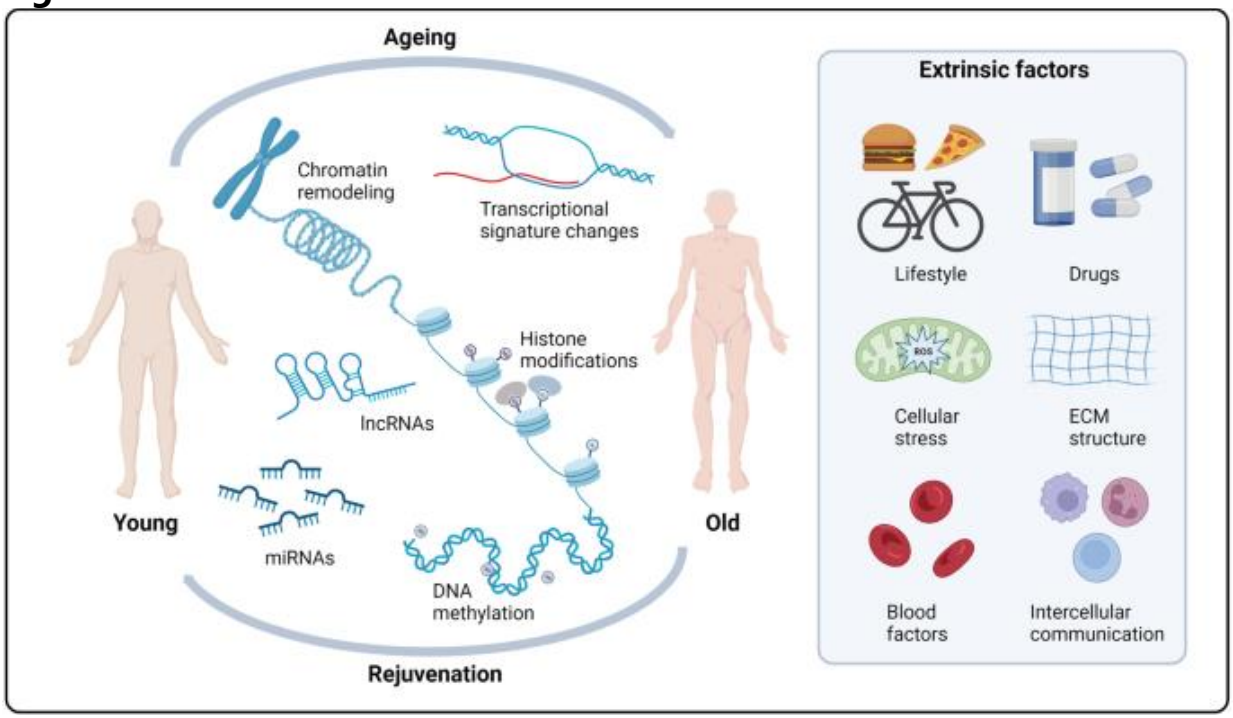
Roadblocks and targets for cellular rejuvenation

Intrinsic barriers limiting cell rejuvenation

Epigenetic alterations and genetic instability

Alterations in physiological and pathological ageing, are frequently caused by disruptions in genetic and epigenetic mechanisms. The universal definition of epigenetics is heritable genomic functionally modifications without DNA sequence variations. Gene expression and chromatin structure are connected with the major epigenetic alterations, which include DNA methylation, histone modifications, and noncoding RNA regulation. Defective transcriptional and chromatin networks have highlighted the contribution to cellular function, stress resistance, and ageing. Thus, epigenetic alterations and genetic instability might affect all cells and tissues in the anti-ageing process, but also provide opportunities for the design of novel rejuvenation treatments (Fig. 2).

Fig. 2



The epigenetic states of ageing and rejuvenation. Ageing and rejuvenation can be affected by intrinsic epigenetic alterations and genetic instability, like DNA methylation and chromatin remodeling. Moreover, many extrinsic factors, like microenvironmental cues, intercellular communication, and systemic factors,

can also impact the epigenetic states of ageing and rejuvenation. miRNAs microRNAs, lncRNAs long noncoding RNAs, ECM extracellular matrix. Created with BioRender.com

Histone variants and modification

Histone variants serve as non-allelic counterparts of canonical histones and are also a common feature in aged organisms. H2A, H2B, H3, and H4 are four core histones variants with different propensity to diversity, regulating specific chromatin regions and gene transcription programs.[10] Numerous research on ageing have demonstrated that histone variations in mammals are associated with a high abundance of macroH2A, H3.3, and H2A.Z.[11] These results all suggested that the histone variants could be potential biomarkers for the ageing state.

Histone modifications include acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination, and proline isomerization, depending on the modification process.[12] These alterations occur at many sites and have various regulatory effects, including DNA repair, DNA replication, transcriptional control, alternative splicing, and chromosome condensation. Most prominently, histone methylation and histone acetylation play crucial roles in epigenetic alterations during ageing. Transcription is activated by the methylation of H3K4, H3K36, and H3K79, whereas it is repressed by the methylation of H3K9, H3K27, and H4K20.[13] Furthermore, numerous investigations have shown that the control of organismal longevity and tissue ageing involves the manipulation of histone methyltransferases or demethylases.[14] Therefore, various histone modifications might be important ageing-associated markers with the potential of anti-ageing drug screening targets.

DNA methylation clock

DNA methylation is widely appreciated as the reversible and inheritable epigenetic mark of the genome, participating in gene expression, biological regulation, and developmental processes in various eukaryotes.[15] This common DNA modification is the synthesis of 5-methylcytosine (5mC), created when a methyl group is added to cytosine in a context involving a

CpG dinucleotide. Long-term alterations in DNA methylation can be influenced by early-life environments and diet, increasing susceptibility to numerous diseases linked to ageing.[16]

Recent studies have discovered the mechanisms causing ageing-related DNA methylation changes and treated DNA methylation as the most promising biomarker of ageing.[17] DNA methylation can directly facilitate transcriptome alterations in cells and tissues and also affect histone modification patterns to regulate gene expression during ageing. Borghesan et al. demonstrated that DNA methylation signatures were the biomarkers of healthy liver ageing and hepatocellular carcinoma progression, and the epigenetic synergism between DNA methylation and histone variant macroH2A1 could control the cancer cell escape from drug-induced senescence.[18] Hence, controlling the DNA or histone methylation machinery might contribute to precise drug delivery. Moreover, certain CpG sites spread across the genome can be used to identify the mammalian DNA methylomes in order to determine the organismal biological age.[19] DNA methylation-based ageing provides an appealing method of pathology prediction due to its continual readout of molecular changes in development. Many rejuvenation interventions, like calorie restriction, dwarfism, and rapamycin therapy, have been shown to slow down the epigenetic clocks and block some ageing-related changes in DNA methylation.[20] Thus, the identification and confirmation of efficient anti-ageing therapies in humans have considerable potential in the DNA methylation.

Nucleosome remodeling

The nucleosome, consisted of pairs of core histones H2A, H2B, H3, and H4 and a DNA strand of 146 base pairs, is the basic unit of chromatin.[21] In an ATP-dependent way, nucleosome remodeling controls DNA repair, replication, recombination, transcription, and cell cycle, as well as the expression of genes important for development and cellular functions in anti-ageing processes.[22] The highly conserved ATP-dependent chromatin remodelers, referred to as sucrose nonfermenting 2 (SNF2) or switch/ SNF (SWI/SNF)-related enzymes, could utilize the energy released during the hydrolysis of ATP, in order to rebuild the chromatin.[23] One particular ATPase might bind

with other types of proteins to form distinct remodeling complexes. ATP-dependent nucleosome remodelers have important roles in modulating lifespan in organisms ranging from yeast to humans.

The modifications of histone core residues perform specific recruitment of transcription factors and remodeling complexes, and also shape nucleosome functions themselves.[24] With increasing age, the nucleosome positioning and occupancy could be inevitably changed, including the loss of core histones and the substitution of canonical histones with variant histones.[25] The availability of DNA for transcription factor binding was proved to depend on different combinations of histone modifications for chromatin remodeling, which also allows the histone code to modify gene expression with ageing.[26] In addition, base excision repair (BER) is a vital DNA repair system for eradicating DNA lesions and maintaining the integrity of the genome.[27] The deficient BER shows a strong link with ageing in human health. Nucleotide excision repair (NER) defects can also affect nucleosome remodeling, histone ubiquitination, stem cell reprogramming, and transcriptional activation, leading to ageing and developmental abnormalities in mammals.[28] Therefore, DNA and histone contents within nucleosomes can go through chemical modifications that change the chromatin conformation and accessibility during ageing process.

Transcriptional signature changes

Numerous transcription factors that control growth, metabolism, and stress resistance are evolutionarily conserved. They are also involved in intricate interactions within various cell types for entire organismal physiologic development and ageing.[29] During ageing process, there are many linked genes to prove the senescence phenotype or type.[30] These biomarkers are linked with the cell-cycle arrest and SASP, such as increased expression of the cyclin-dependent kinase inhibitors p16 and p21, reduced expression of the nuclear lamina protein LaminB1, increased secretion of many inflammatory cytokines, and others. Many transcriptional responses to oxidative stress and pathogens decrease with ageing, owing to the declining function of the stress-responsive NF-E2-related factor 2 (Nrf2).[31] Forkhead box O (FOXO) transcription factors are also revealed as critical mediators in crucial cellular

processes in mammals during ageing.[32] Thus, ageing was accompanied by both increased transcriptional instability and an accumulation of genetic errors. Besides, RNA N^6-methyladenosine (m^6A) modification has uncovered a new domain in post-transcriptional epigenetic regulation. In intervertebral discs (IVDs) degeneration, the m^6A and the crosstalk between m^6A and histone/DNA modifications have been proved to facilitate nucleus pulposus cellular senescence and aggravate cartilage endplate degeneration.[33] To determine the diverse biological senescence, the transcriptome signatures associated with senescence and variability of the senescence program contribute to the identification of particular senescence biomarkers in tissues and organisms.

Noncoding RNA profiles

Noncoding RNAs (ncRNAs) are a broad and diverse group that produce non-protein-coding transcripts, including micro RNAs (miRNAs), long noncoding RNAs (lncRNAs), and circular RNAs (circRNAs). Several studies have discussed that the ncRNAs are able to bond to DNA, RNA, and protein, influencing cellular proliferation, quiescence, differentiation, apoptosis, and senescence.[34] Specifically, ncRNAs can strongly implicate in controlling senescence at transcriptional, post-transcriptional, and post-translational stages. Especially, miRNAs are the best characterized small ncRNAs influencing ageing and lifespan. Multiple miRNAs, including miRNA-1, miRNA-145, miRNA-140, miRNA-34a, miRNA-106b, and miRNA-449a, are widely considered as critical regulators for cell senescence.[35] They impact the SASP phenotype and modulate senescence through the classical p16, p53, and calcium signaling pathways.[35] Furthermore, the lncRNAs expressions are known as a result of the disease-triggering stimulation in reduced cardiovascular vigor and cardiovascular ageing. There are increasing lncRNAs as indications of a deteriorating prognosis following cardiac events, such as the lncRNA MIAT, ANRIL, LIPCAR, and MALAT1.[36] More importantly, the potential of circRNAs to serve as miRNA sponges and the interacting regulatory network between lncRNAs and miRNAs are all connected to ageing-related changes and the switching of the cell fate.[37] In addition, extracellular RNAs in circulation and other bodily fluids also play vital roles within the context of ageing. The pericentromeric ncRNAs can be transported

into neighboring cells via extracellular vesicles (EVs), and impaired the DNA binding of the CCCTC-binding factor to modify chromosomal accessibility and trigger an SASP-like inflammatory response.[38]

Macromolecular damage

The specific complex of cell macromolecules, including telomeres, proteins, and lipids, possesses intrinsically high resistance to modification, contributing to superior longevity in species. Reducing macromolecular damage is associated with an improvement in the majority of ageing-related physiological activities. Thus, the single macromolecular regulator of ageing and the interconnectivity among molecular phenotypes can both induce alterations in ageing-related phenotypes, limiting cell rejuvenation.

Telomere attrition

Telomeres are repetitive DNA sequences at chromosome ends. Telomerase adds repeats to the ends of the chromosomes during genome replication to counteract the loss of telomeric DNA. In most eukaryotes, the telomerase-based mechanism for telomere preservation is crucial for genomic stability and cell viability. The compaction of telomeric chromatin robustly protects the ends by limiting the accessibility of the DNA damage response machinery.[39] However, telomeres are gradually shortened with cell division, and the cells enter a replicative senescence state when they cannot effectively guard the ends of the DNA.[40] DNA damage accumulation with age also affects the genome randomly. Telomere dysfunction-induced foci and telomere-associated foci are two types of DNA damage that specifically target telomeres.[41] Significantly short telomeres or changed telomere architecture can result in dysfunctional telomeres in ageing-associated pathology.[42] This telomere dysfunction is closely associated with telomeropathies, telomere biology disorders, and telomere syndromes. In addition, ncRNAs also emerge as key inducers of telomere length maintenance in senescence and ageing-related diseases.[43]

Loss of proteostasis

Proteome homeostasis is maintained by the proteostasis network (PN), a macromolecular system that coordinates protein synthesis, folding,

disaggregation, and degradation for organismal health and longevity. The autophagy-lysosomal system and the ubiquitin-proteasomal system (UPS) are two crucial mechanisms that regulate the turnover of organelles and aggregates.[44] Proteasomes are also charged with removing normal and damaged proteins, participating in the evolutionarily conserved ageing mechanism and longevity regulation. However, ageing often shifts the balance between the protein lifecycle in organisms, resulting in pathology. UPS dysregulation occurs in the ageing process and several ageing-related diseases in mammals.[45] PN component aggregation during ageing can elicit aberrant transcriptional procedures, reduced folding capacity, and the accumulation of misfolded species.[46] This loss of proteostasis might further have profound consequences for ageing progression and age-related disease presentation. In addition, the increased ribosome pausing during ageing can also make the ribosome-associated quality control overloaded, leading to proteostasis impairment and systemic decline.[47]

Lipid Damage

Lipids are crucial components of all cell types, and perform various biological functions, including energy storage, cell membrane construction, signal transduction, protection, and mitochondrial regulation. There are a variety of bioactive lipids with critical roles in influencing cell age and the progression of several age-associated diseases and metabolic abnormalities.[48] Specifically, the lipid assemblies act as scaffolding for the construction and function of signaling complexes and play critical roles in the preservation of proteostasis, thus involving in the ageing process and neurodegenerative disease development.[49] Cholesterol, phospholipids, ganglioside GM3, and sphingomyelin are lipid classes that are typically present in cell membranes, playing their respective roles in membrane fluidity and rigidity. The lipid-induced changes in membrane structure and remodeling of lipid composition are the causative agents of ageing phenotypes.[50] Moreover, many pathways regulating ageing and longevity are also linked to lipid metabolism and lipid signaling. According to research on the serum and plasma lipidome in centenarians, descendants of centenarians and elderly people without age-related disorders, the lipid signature profile altered with ageing.[51] It also showed changes in antioxidant capacity, lipid peroxidation levels, and

inflammation along with modifications in lipid metabolic pathways with ageing.

Metabolic imbalance

Age-dependent alterations in the transcriptomes, proteomes, and metabolomes of different organisms and tissues reveal the imbalance of metabolic homeostasis. Remodeling of metabolic signals and metabolites in ageing and the control of lifespan is caused by organelle malfunction, redox imbalance, and changed signaling pathways.[52] Both environmental and generated endogenous toxicants by metabolism are major contributors to macromolecular damage and physiological dysregulation during ageing. The metabolic phenotyping of ageing mice revealed the involvement of the adiponectin, growth hormone, and cytokine pathways in autophagy, stress response, genome integrity, mitochondrial biogenesis, energy balance, inflammation, and infection control.[53]

The main mechanism to foster ageing is the malfunctioning of vital cellular organelles, including the autophagosomal-lysosomal network and mitochondria. With advancing age, there is a reduction in autophagy activity, autophagosome production rate, and lysosome fusion activity.[54] Insufficient protective autophagy during ageing might cause damaged cellular components to accumulate and dysfunction of cellular organelles, leading to metabolic imbalance and further ageing. Moreover, defective mitochondrias produce insufficient ATP and frequently produce more ROS to enhance oxidative stress.[55] Aged cells commonly develop mitochondria with aberrant characteristics, such as point mutations and mitochondrial DNA (mtDNA) deletions.[56] In addition, mitochondrial metabolism also includes carbon metabolism (the tricarboxylic acid (TCA) cycle), the biosynthesis of Fe/S clusters, and the metabolic consequences of mitophagy.[57] These metabolic processes are highly dynamic and all influence different facets of ageing.

There are metabolic enzymes and pathways to maintain homeostasis, including acetyl-coenzyme A (acetyl-CoA), pyruvate, 2-oxoglutarate, glycolysis, the TCA cycle, the urea cycle, respiration, and oxidative phosphorylation.[58] The dysfunctions of these metabolic enzymes and mechanisms trigger metabolic

disorders and restricted lifespan. Many core metabolites are appearing as key regulators of ageing, including nicotinamide adenine dinucleotide (NAD^+), reduced nicotinamide dinucleotide phosphate (NADPH), α-ketoglutarate (α-KG), and β-hydroxybutyrate (βHB).[59] A change in the NAD+/NADH ratio or the size of the NAD+ pool can cause the biological system to malfunction and result in a variety of metabolic diseases, ageing, and cancer.[60] In addition, the telomere shortens and telomerase dysfunction might downregulate peroxisome proliferator-activated receptor gamma coactivator (PGCs) and other metabolically relevant genes, which are linked to hampered mitochondrial biogenesis and function, reduced gluconeogenesis, cardiomyopathy, and elevated ROS.[61] It has been demonstrated that altering the insulin/IGF-1 and mammalian target of rapamycin (mTOR) signaling pathways significantly slows down the ageing process in a variety of species.[62] Metabolic interventions, like time-restricted feeding, ketone bodies, rapamycin, metformin, resveratrol, NAD boosters, glycolytic inhibition, mitochondrial-derived peptides, and poly (ADP-ribose) polymerase (PARP) activators, all target these conserved pathways and biological ageing mechanisms across species, to boost adaptability, rehabilitation, and postponed ageing.[63]

Extrinsic factors impacting cellular rejuvenation

Local microenvironmental cues

Extrinsic cues are transmitted by different stromal cell types within their niche and tissues, resulting in an actively responsive microenvironment. The ECM, neighboring cells, and signaling molecules, such as hormones, growth factors, and metabolic products, all play roles in mediating the interaction between the cell and the microenvironment. With time, the microenvironment that maintains multicellular organization is chronically altered, which further remodels the intracellular processes and induces ageing and cancer development.[64]

MSC lineage shift in ageing is mostly caused by microenvironmental effects. The crucial microenvironmental cues that cause differentiation abnormalities in MSCs are caused by hormonal, immunologic, and metabolic variables. For

example, BMSCs in ageing could misdirect the differentiation toward adipocytes to impair osteogenesis, leading to the pathogenesis of osteoporosis.[65] Especially, bone-fat reciprocity and the development of mesenchymal progenitors toward an adipogenic fate were mostly caused by the microenvironmental changes that occurred with in vivo ageing.[66] Therefore, distinct microenvironmental conditions are deciding on cell fate, including exogenous growth factor stimulation, adjacent cells communication, pH, osmolarity, oxygen concentration, temperature, air pressure, biomechanical and electromagnetical influence.[67] These microenvironment changes might increase the difficulty for normal cells to maintain homeostasis and react to damage.

Age-associated changes in ECM structure and composition

As the three-dimensional macromolecular network without cells, the ECM is primarily made of an interconnected system of fibrillar and non-fibrillar collagens, elastic fibers, and glycosaminoglycan-containing non-collagenous glycoproteins (hyaluronan and proteoglycans). Certain enzymes that stimulate ECM destruction, like matrix metalloproteinases (MMPs), mediate the ECM remodeling process.[68] ECM maintains tissue integrity, and its dysregulation during ageing leads to various disease disorders by altering its composition, morphology, rigidity, and abundance. Many ECM genes and remodelers can be directly regulated by the mTOR signaling pathway, SIRTs, and numerous longevity-promoting transcription factors, such as KLF4, MYC, and HIF1, which control ECM dynamics during ageing.[69]

The ECM is necessary for normal tissue repair, but excessive deposition can cause organ malfunction and the onset of fibrotic and degenerative diseases. Especially, the adult dermis quality following complete maturation gradually deteriorates with age, such as atrophy of the elastic network, disintegration of collagen fibers, and alterations modifying proteoglycans.[70] Moreover, ageing causes localized flaws and superficial fibrillation of the articular surface to accumulate. For instance, osteoarthritis (OA) might occur due to the altered matrix component composition, declining water content in the tissue, and increased catabolism in the ECM.[71] Age-dependent functional deficits of muscle stem cells (MuSCs) are attributed to extensive ECM remodeling during

ageing.[72] In cancer, the main biochemical, physiological, and mechanical factors associated with ageing ECM promoted invasive and cancer-like activity in both healthy and malignant cells.[73]

Altered intercellular communication

Intercellular communication networks are essential for the coordination of biological processes in healthy and pathological settings of multicellular organisms. Senescence and ageing are influenced by interferences with intercellular communication caused by metabolic, mechanical, or biochemical triggers. To sustain physiologic function and respond to diseases, the many cell types that form the neurovascular unit (NVU) are in constant contact. The insufficient crosstalk between NVU cells impairs neurovascular coupling and blood-brain barrier dysfunction, thus leading to ageing and related neurological and neurovascular diseases.[74] Moreover, there are also many organelle-organelle and organelle-cytosol communications impacting chronological ageing. These communications form an intricate network involving various movements of metabolites between cellular compartments. The process of stem cell ageing and tissue and organ functional declining is attributed to mitochondrial-ER crosstalk.[75] Age-related diseases and the ageing process are linked to aberrant EV secretion and disturbance of the mitochondrial-lysosomal axis.[76]

Senescent cells are extremely proactive and interact with nearby cells through a variety of intercellular channels, including SASP. As the traditional soluble SASP, soluble factors, growth factors, and matrix remodeling enzymes are released. Intercellular communication during senescence via receptor or cell-ECM interaction is referred to as nonclassical SASP, and emerging SASP components include EVs.[77] Furthermore, EVs are released into extracellular space and act as a cell-to-cell means of communication. EVs have negative impacts on downstream effectors at the levels of immunology, inflammation, gene expression, and metabolism in the ageing setting and age-related illnesses.[78] Aged and senescent cells are proved to release more EVs than young cells.[79] In addition, several environmental conditions, including air pollution, ultraviolet light, nutrition, and physical exercise, have been verified to impact the communication network via EVs, further impacting ageing.[80]

Systemic factors

Alterations in the systemic environment of cells and tissues play a role in the reversible process of ageing. Organ dysfunction with ageing is caused by blood-mediated cell-extrinsic alterations and important molecular mechanisms in the systemic environment. One of the cell types that reacts to young blood exposure is the hematopoietic stem cells (HSCs).[81] The hematopoietic and immunological systems can be rejuvenated by the young transcriptional regulation system and cytokine-mediated cell-cell interactions in HSCs. Many agonists and antagonists of specific signaling pathways have the effective capability of resetting tissue stem cells in aged organs into rejuvenating state.[82] In addition, systemic obesity, air pollution, exercise, and psychological stress have been clarified to accelerate ageing at molecular and epigenetic levels.[83] Some biological techniques, such as heterochronic transplantation and parabiosis, might give cells and substances that are more abundant in young individuals to recover the function of aged tissue. Heterochronic parabiosis is the surgical method of young and aged organisms using a common vascular system, showing the significant impact of the systemic environment on ageing and rejuvenation. Many studies on neurogenesis have reported the pro-neurogenic "youthful" factors in the circulation and "ageing" substances that reduce stem cell activity in heterochronic parabiosis models of young and aged mice.[84] Moreover, systemic transplantation of stem cells also displays the therapeutic potential of preventing age-associated degeneration. However, the systemic and hormonal changes with age, including pro-inflammatory cytokine profiles and sex steroid changes, also influence stem cell transplantation effectiveness.[85]

Rejuvenating-targeted cells for organismal youthful state

Stem cells

The major types of stem cells include adult stem cells, embryonic stem cells (ESCs), and iPSCs created by activating Yamanaka factors from various somatic cells. They have the unique capacity for self-renew and multipotency, and can differentiate into tissue-specific terminal cell types. MSCs are pluripotent cells developed from adult stem cells. Many studies have demonstrated that MSCs produced from various sources, such as bone marrow, adipose tissue, and

umbilical cord blood, and MSC-derived compounds slowed ageing process and improved age-related conditions.[86] Tissue stem cells are found in particular local tissue microenvironments called "stem cell niches," which support stem cell maintenance. Tissue stem cells play key roles in facilitating organic tissue renewal and performing regenerative responses to injury.[87] ESCs can self-renew and differentiate into multiple cell types of ectoderm, endoderm, and mesoderm lineages.[88] More importantly, the iPSCs modified from autologous sources also have ESC-like states and pluripotent potential. iPSCs transform patient-specific samples from early cells into developed target tissues, showing potential for age reversal within the organism.[89] These stem cells are excellent rejuvenation targets and all have promising potential in regenerative medicine.

With increasing age, decreased stem cell functionality can lead to diminished organ function and prolonged tissue repair. Targeting the age-related molecular basis of stem cells might reduce the deleterious effects of ageing. There are many rejuvenating approaches based on aged stem cells, such as delayed fasting, gene expression modulation, medicinal intervention, and niche changes.[90] However, Ho et al. found that some rejuvenating approaches had no observable renewed effects on aged HSCs and aged bone marrow niches.[91] Some rejuvenation techniques might show temporary benefits, but show harmful long-term effects by prematurely diminishing the stem cell pool.[92] Thus, it is also vital to strike a balance between the regenerative properties of stem cells and their potential to induce cancer.

Vascular and connective tissue cells

Endothelial cells (ECs) and smooth muscle cells (SMCs) are critical building blocks of blood channels and are negatively impacted by premature or typical ageing processes. In ageing process, dysfunctional ECs and endothelial progenitor cells (EPCs) occur abnormal metabolism, the development into mesenchymal phenotype, vascular detachment, and myofibroblast formation, resulting in fibrosis and organ dysfunction.[93] ECs and other neurovascular cells are key cells in maintaining blood-brain barrier function. Dysfunction of pericytes, astrocytes, and endothelial cells increases blood-brain barrier permeability during ageing.[94] The intricate biological process of targeted EC

regeneration involves migration, survival, proliferation, tube formation, and restoring blood flow to the ischemic organs for tissue homeostasis. EPCs might be obtained from the bloodstream or niches within the vascular wall and restored by the ectopic production of mediators that prevent senescence and the onset of ageing-related traits.[95] Thus, targeting the endothelium via regulating the senescence-induced gene expression and other emerging rejuvenation mechanisms is essential for homeostasis and tissue regeneration.

The major stromal cell type is the fibroblast, which regulates tissue morphology by depositing ECM, and promotes cellular and microenvironmental homeostasis by secreting soluble substances and signaling proteins. During the ageing process, fibroblasts lose contractility and exhibit an unbalanced production and degradation of ECM proteins, ultimately leading to reduced connective tissue stiffness and even age-related diseases.[96] Activated fibroblasts and senescent fibroblasts secrete inflammatory cytokines with a different ratio, affecting complex reprogramming and wound healing in mice.[97] Many studies have insisted that some anti-ageing compounds like triacetylresveratrol and cannabidiol, gene expression, signaling regulation, and cell-cell communication are all effective methods for targeting fibroblasts for rejuvenation, longevity, and health.[98] Hence, as a cell type commonly used for iPSC reprogramming, fibroblasts are essential targets for rejuvenation techniques and regenerative medicine.

Senescent cells

Senescent cells (SCs) comprise a heterogeneous cell population because of their various cell-autonomous activation pathways and microenvironmental circumstances. Although cell-cycle arrested, SCs are still metabolically active and can perform various functions of the parent cells.[99] At present, SCs, as organismal carriers of irreparable damage, are identified by the senescence-associated gene expression, SASP production, DNA damage, and β-galactosidase activity.[100] MSC populations with a high number of SCs are found less productive during transplantation.[101] The excessive accumulation and activity of SCs are also linked with chronic ageing and age-related illnesses, including atherosclerosis, cardiac and kidney dysfunctions,

neurodegeneration, and pulmonary fibrosis.[102] SCs are also able to trigger senescence in non-senescent cells. High quantities of SCs secreting chronically SASP are found in aged tissues, causing irreversible reprogramming of their adjacent cells.[103] Hence, it also needs to prevent the subsequent development of SCs after the emergence of initial SCs. Partial reprogramming of SCs can reduce the persistent inflammatory state related to ageing and secondary senescence in surrounding cells by inducing the SASP.[104] The specific gene expression on the surface of SCs might lead to the advancement of senolysis techniques for selective elimination.[105]

Immune cells

During ageing, the immune system progressively undergoes disorders of immune cell generation, differentiation, and function, leading to a chronically subclinical inflammatory condition. Several studies have proposed that targeting central immunological processes and specific immune subpopulations can reduce specific age-induced immune changes.[106] Neutrophils are abundant immune cell populations in early injury and serve numerous functions in tissue regeneration. Macrophages reside in the bone marrow and are defective in efferocytosis and hyperactivated with ageing. Neutrophils can also function as an anti-inflammatory shift in macrophages by influencing the surrounding microenvironment or controlling the behavior of macrophages during tissue injury.[107] The deficiency of immunosurveillance might hamper the SCs clearance and induce a microenvironment of chronic inflammation, leading to pro-tumorigenic events.[108] Therefore, enhancing the immune surveillance ability of macrophages is also an effective rejuvenation target, due to the macrophage function of selectively SCs recognition and elimination.

The senescence in immune cells affects innate and adaptive immunity, particularly natural killer (NK) cells, B cell, and T cell function, potently driving age-related changes in solid organs. In vivo reprogramming might be significantly impeded by NK cells, which identify and eliminate partially converted cells in a degranulation-dependent mode.[109] T cell generation is decreased because of thymic involution. Some hormones, signaling pathways, cytokines, and growth factors might display T cell reconstitution effects and

reduce the negative effects of age-related T cell deficiency.[110] Adoptive cell transfer of naive T cells can promote immunological responsiveness to new antigenic stimuli and limit the growth of pathogenic memory T cells.[111] However, the quantity of naive T cell reconstitution required to boost immunological defense in ageing organisms still has quantitative constraints.[112] Furthermore, B cell production also decreases with age due to the reduction of hematopoietic bone marrow. An in vitro B cell population with youthful characteristics and cellular reactivity to immunological stimulation can be revived after B cell depletion in elderly mice.[113]

Other somatic cells

Many specialized cells with different sources deserve further research for tissue and organ regeneration and rejuvenation. For example, in the vertebrate retinas, Müller cells serve as the primary supportive and protective glial cells. They can secrete various cytokines and exhibit the potential for self-renew and trans-differentiation into retinal neurons.[114] Pathological ageing might impair β-cell function in the pancreas, thus causing the imbalance of glucose homeostasis in the organism. Targeting β-cell and restoration of function is of vital importance for effective therapeutic strategies.[115] At present, there are emerging reprogramming strategies conversing differentiated somatic cells into another cell type. For instance, the astrocytes and pancreas exocrine cells can be respectively direct reprogrammed into neuroblasts and β-cells via lineage-specific transcription factors.[116] Thus, these cells and related genes and pathways might facilitate the target-based gene delivery and development of effective rejuvenation approaches.

Common or distinct mechanisms of rejuvenation

Signaling pathways

There are various signaling pathways identified in the fields of ageing and rejuvenation, such as nutrient-sensing pathways, DNA damage pathways, ROS and mitochondrial unfolded protein response (UPR^{mt}) pathways, inflammation-related pathways, transforming growth factor-β (TGF-β) pathways and Wnt/β-catenin pathways. The known role of these signaling pathways is complex and mutually connected. Given the prominent association of signaling pathways with rejuvenation and ageing, targeting these signaling systems

pharmacologically and therapeutically has great potential for rejuvenation and human health (Fig. 3).

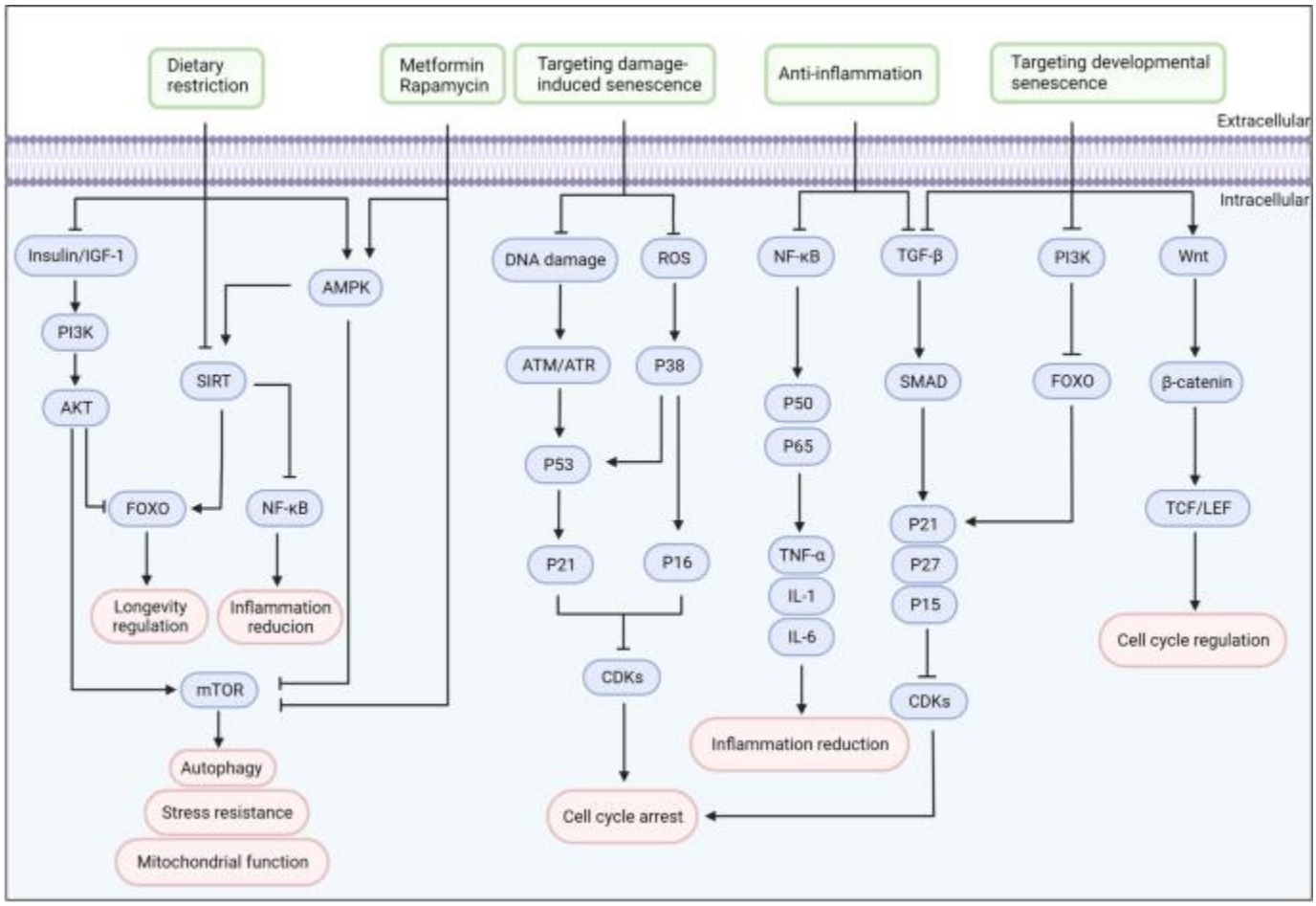


Fig. 3

The target signaling pathways for cellular rejuvenation. Target signaling pathways for cellular rejuvenation are listed according to their biological functions. Many interventions, like dietary restriction and drugs, improve metabolism and extend longevity through nutrient-sensing pathways. In addition, targeting the pathways of damage-induced and developmental senescence can regulate the cell cycle and alleviate age-associated phenotypes. Modulating many inflammation pathways also provides an effective route to rejuvenation. IGF-1 insulin-like growth factor 1, PI3K phosphoinositide 3-kinase, AKT protein kinase B, AMPK 5′-AMP-activated protein kinase, NF-κB nuclear factor-κB, STAT3 signal transducer and activator

of transcription 3, mTOR mammalian target of rapamycin, SIRTs sirtuins, FOXO forkhead homeobox type protein O, ROS reactive oxygen species, TGF-β transforming growth factor-β, CDKs cyclin-dependent kinase. Created with BioRender.com

Nutrient-sensing pathways

Nutrient availability is crucial in regulating ageing and rejuvenation in mammals. Many growth factors, metabolites, amino acids, and carbohydrates can be recognized by several proteins, such as insulin/IGF-1, mTOR, SIRTs, and AMP-activated protein kinase (AMPK). The insulin/IGF-1 signaling pathway is an evolutionarily conserved glucose sensors mechanism, involving developmental defects and decreased adult functionality with age.[117] The insulin/IGF-1 pathway participates in the regulation of many cellular functions, including the metabolism of lipids and carbohydrates, the cellular availability of glucose, gene expression, and cell differentiation, growth, and survival. The stimulation of insulin/IGF-1 receptors can stimulate STAT3 signaling via Janus kinase (JAK) and protein kinase B (AKT)-driven signaling pathways, then forming negative feedback to inhibit insulin/IGF-1 and induce cell immune senescence.[118] The insulin/IGF-1 binding to membrane transporters can stimulate the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway, followed by the downstream FOXO1 phosphorylation and mTOR upregulation, hence regulating cellular ageing and rejuvenation.[119] Furthermore, the mTOR is also a conserved nutrient-sensing protein kinase that regulates eukaryotic cell growth and metabolism. There also remain AMPK/mTOR and PI3K/AKT/mTOR pathways implicated in the accumulation of unfolded and misfolded proteins and the regulation of the autophagy process during ageing.[120] DR and rapamycin have been proved to inhibit the mTOR signaling pathway causing the downregulation of cell growth and lifespan extension.[121] Hence, the insulin/IGF-1 and mTOR pathways are promising targets to suppress or delay ageing-associated diseases and extend lifespan.

SIRTs are a family of seven paralogous NAD^+-dependent enzymes, that control cell proliferation, energy metabolism, stress tolerance, inflammation, circadian rhythms, neural function, and ageing.[122] The activity of SIRTs in many or combinations of tissues might be necessary for lifetime extension and

rejuvenation. Meanwhile, the AMPK pathway is a core mediator of energy homeostasis engaged in the pathobiology of ageing and age-linked disorders.[123] AMPK is also considered as a crucial integrator of inflammation-controlling signals, including the inflammasome.[124] It is well known that among different interventions to promote longevity, like exercise, intermittent fasting and DR, nutrient supply reduction activates AMPK to promote ATP production.[125] Metformin can activate AMPK and SIRT1 and downregulates insulin/IGF-1 and mTOR, thus playing beneficial roles in energy metabolism and ageing.[126] In general, the above nutrient-sensing pathways and the interaction between SIRT, AMPK, and mTOR all explain a mechanism for rejuvenation.

DNA damaging pathways

The cell functionality can be hampered by persistent DNA damage, which can also accelerate senescence and apoptosis.[127] DNA damage response (DDR) pathway can protect against endogenous and exogenous damage and ensures the integrity of the genome.[128] In response to stress, the established DDR contributes to the stimulation of p53 and p16 pathways and the depression of cyclin-dependent kinase (CDK) inhibitors to initiate and sustain the arrest of cell cycle.[129] The genetic mutations that augment DDR and persistent DNA lesions can continuously activate the DDR. Moreover, the alterations in the integrity and efficacy of mtDNA repair contribute to DNA damage accumulation, illness, and ageing.[130]

The cytosolic DNA sensor cyclic GMP-AMP Synthase (cGAS) binds to the effector protein stimulator of interferon genes (STING), initiating a DNA sensing signaling pathway for innate immune responses.[131] Misplaced cytosolic self-DNA and alteration of mitochondria structure and function can activate cGAS-STING signaling pathway, thus constituting age-related inflammation.[132] The DNA mismatch repair system (MMR) is largely conserved across organisms and is essential for maintaining DNA integrity.[133] Hence, MMR and related repair proteins are necessary for lifespan extension and rejuvenation targets. There are also a series of systems repairing the DNA, including BER, NER, and double-strand break (DSB).[134] Thus, multiple interventions have been developed to lessen the DNA damage accumulation

and alleviate age-associated phenotypes, such as lowering destructive molecules, restoring DNA damage, and responding to persistent DNA damage.

ROS and UPR^{mt} pathway

Reactive oxygen species (ROS) are mainly generated from oxidative phosphorylation in mitochondria, and maintain a dynamic balance with antioxidation systems under physiological conditions. Low ROS levels enhance the defensive mechanisms by producing adaptive responses for stress tolerance and longevity, whereas high ROS levels create insufficient adaptive responses that may accelerate the onset and course of ageing.[135] The key mediators of the ageing process are ROS and ROS-induced oxidative damage produced by cellular metabolic and respiratory processes. Besides, mtDNA is susceptible to damage by mitochondrial ROS.[136] Thus, the ROS causes oxidative stress, damages mitochondria, and induces energetic obstacles that lead to accelerated ageing and various diseases. Peroxiredoxins have been shown to facilitate ROS-based redox signaling and to trigger many cellular stress responses.[137] However, these beneficial effects of ROS might be proved to be a sign of toxic adaptation.[138] Interventions to ROS pathways are widely proposed as anti-ageing and rejuvenation strategies.

Metabolic stress, hypoxia, protein damage, and mitochondrial ROS all can impair mitochondrial protein homeostasis and functions. The transcriptional activation program of mitochondrial chaperone proteins and proteases is known as the mitochondrial unfolded protein response (UPR^{mt}), which is a mitochondrial response to stress.[139] Studies have shown that UPR^{mt} plays a significant role in many physiological processes and that its activation increases longevity and prevents ageing by regulating mitochondrial proteostasis.[140] During mitochondrial dysfunction, there are many transcription factors necessary for the activation of UPR^{mt} genes in mammals. Activating transcription factor associated with stress-1 (ATFS-1) participates in the upregulation of genes involved in multiple stress response pathways for organismal survival of acute stressors.[141] But, chronic ATFS-1 activation also has a negative effect on longevity. Meanwhile, in the initial development stages of elderly individuals, age-dependent levels of histone 3 methylation

partially influence UPR^{mt} activation.[142] UPR^{mt}-mediated protective mechanism might be beneficial for rejuvenation mechanisms and therapeutics for diverse metabolic diseases and ageing-related disorders. However, prolonged UPR^{mt} activation might also induce the propagation of mitochondrial damage.[143] The mitochondrial permeability transition pore in the inner mitochondrial membrane, can also initiate UPR^{mt} to promote ageing and age-related diseases.[144]

Inflammation-associated pathways

Multiple signaling cascades whose integration targets the induction of senescence, ageing, and associated disorders can be activated by the cycle of physiological interactions between inflammation and oxidative stress. In mammals, the major pro-inflammatory cytokines, which include interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and IL-1α, significantly remodel the immune system.[145] Damage-related stimuli cause the release of danger-associated molecular patterns (DAMPs) with ageing, and DAMPs that activate TLRs or the NLRP3 complex can be involved in sterile inflammation and age-related diseases.[146] These age-related increases in chronic and low-level sterile inflammation are known as “inflammageing”, which shows a strong occurrence of various age-related diseases more than with ageing itself.[147]

The NF-κB signaling pathway is highly linked with the initiation and deterioration of tissue inflammation and ageing process. NF-κB can induce pro-inflammatory mediators, SASP, chemokines, and adhesion molecules, and the crosstalk between upstream signaling elements including MAPK, mTOR, and protein kinase B affects the transcriptional activity of NF-κB.[148] Amplification loops for inflammatory processes are created when pro-inflammatory cytokines activate NF-B, which in turn can produce additional cytokines.[149] The chronic activation of pro-inflammatory NF-κB/Rel and JAK/STAT signaling pathways contributes to the declined regeneration potential in mouse models.[150] The JAK2 gene mutations in hematopoietic ageing can trigger abnormal involvement of downstream signaling pathways and the establishment of an inflammatory environment.[151] Overactive JAK signaling is considered as a signature of immune disorders and has a significant impact on inflammation, coagulation, and thrombosis. Therefore,

these shared pathways might provide a common route to rejuvenation by modulating inflammation, and some distinct pathways have great potential of targeting improving specific functions.

TGF-β signaling pathway

The transforming growth factor beta (TGF-β) superfamily is a vast protein group, including three TGF-βs (TGF-β1–3), bone morphogenetic proteins (BMPs), and growth differentiation factors (GDFs).[152] The TGF-β family play roles through heteromeric combinations of type I and type II receptors, thereby activating many signal transducers, containing SMAD-dependent, SMAD-independent, and non-SMAD signaling pathways.[153] These signaling pathways perform multiple functions in the development of embryo, tissue homeostasis and repair, immunological responses, tumor suppression, and metastasis. Many findings on age-related diseases have reported that TGF-β signaling dysfunction or increased levels of TGF-β ligands induce metabolic dysfunction, tissue fibrosis, inflammation, regeneration suppression, and cell degeneration.[154] Besides, TGF-β signaling can induce specific epigenetic alterations further promoting senescence and ageing. Meanwhile, ageing also induces many abnormalities at the TGF-β receptor level. TGF-β signaling possesses dual functionality and versatility in some age-related disorders and cancer as a suppressor and a promoter.[155] Hence, many strategies targeting TGF-β are mainly focused on inhibition of production, activation, binding to the receptor, and intracellular signaling.

TGF-β signaling can regulate matrix protein synthesis and matrix degradation, and alter cell-cell interaction. TGF-β overexpression results in ECM deposition, epithelial–mesenchymal transition (EMT), and cancer-associated fibroblast (CAF) formation, then leading to fibrosis and cancer.[156] More importantly, dysregulation of the TGF-β/SMAD pathway is reported as an essential causative agent in tissue fibrosis, like hepatic, pulmonary, and cardiac fibrosis.[157] TGF-β modulates several intracellular signaling cascades to deliver profibrotic effects, and the numerous ways of TGF-β interacting with other profibrotic pathways all offer the potential for therapeutic intervention.[158] Furthermore, TGF-β signaling regulates the levels of angiogenesis-related molecules, like VEGF and CTGF, and mediates immune

regulation, inflammation, and other pathways.[159] In addition, GDF11, one cytokine of the TGF-β superfamily, is identified as a rejuvenating element in neurodegenerative and neurovascular diseases, such as the reversal of senescence and age-related variations, and the modulations of organ regeneration after injury.[160]

Wnt/β-catenin signaling pathway

Wnt proteins are secreted glycosylated proteins, which are cysteine-rich and can initiate the transcriptional co-activator, β-catenin, leading to the target gene upregulation via the family of T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors.[161] By building a stable compound with the cell adhesion molecules of cadherin family, β-catenin, a transcription cofactor with dual roles, participates in cell adhesion.[162] The Wnt/β-catenin signaling pathway participates in the modulation of genetic stabilization, cell proliferation, migration, and apoptosis for developmental processes and tissue homeostasis.[163] The transcriptional results of Wnt/β-catenin pathway activation change with various cell types. The canonical Wnt/β-catenin pathway as the core mechanism is essential for directing stem cell regeneration and differentiation. For preservation and transition from the pluripotent state during embryo development, stem cells need β-catenin to moderate the response to Wnt signaling.[164] Wnt/β-catenin signaling can also regulate the expression of telomerase reverse transcriptase (TERT) and change the telomere length, thereby affecting stem cells, ageing, and cancer.[165] Wnt/β-catenin signaling can serve as a promising target to ameliorate the deterioration of stem cell function.

Research findings have verified that Wnt/β-catenin signaling regulates the ageing process of several tissues, performing different changes in different organs. Increased Wnt signaling has been found in aged organisms and excessive levels of Wnt are damaging to organism functionality.[166] Wnt/β-catenin pathway is crucial for the growth and development of mineralized tissues, for the regulation of the skeleton in respond to loading and unloading, and for maintaining the viability and fitness of the adult and ageing skeleton.[167] More importantly, the dysregulation of Wnt/β-catenin pathway is responsible for the fibrotic tissues associated with ageing, such as kidney, liver,

lung, and heart fibrosis.[168] Wnt/β-catenin signaling and TGF-β signaling can interact in the fibrosis process. Wnt/β-catenin superfamily members can be activated by TGF-β signaling and vice versa.[169] Meanwhile, synaptic assembly, neurotransmission, and synaptic plasticity are all regulated by Wnt ligands, and neurodegenerative diseases are linked to deregulated Wnt signaling.[170] Therefore, manipulating Wnt/β-catenin pathway might promote an efficient rejuvenation strategy versus ageing.

Reprogramming-induced rejuvenation

Rejuvenation by induced pluripotent stem cells

iPSC reprogramming is widely defined as the rejuvenation of mature differentiated cells to an embryonic-like fate.[171] By forcing the expression of specific transcription factors, induced pluripotency is the potent capacity to differentiate into all cell types. The transcriptome, epigenome, and metabolome of differentiated cells can be significantly altered by the transient expression of Yamanaka factors (OCT4, SOX2, KLF4, and cMYC; OSKM), which can also remodel the cells into iPSCs.[172] The most common cell types for reprogramming to iPSCs are fibroblasts, which are greatly implicated in regenerative medicine and rejuvenation strategies. But in distinct subpopulations of fibroblasts, the change of fibroblast component and the level of secreted inflammatory cytokines might affect the in vitro reprogramming effectiveness and in vivo wound healing rate.[173] In addition, the generation of iPSCs can realize the rejuvenation of MSCs and the iPSCs differentiation into desired cells via variations in DNA methylation, histone composition, and epigenetic models.[174] Therefore, the iPSCs applications can minimize the genetic and epigenetic abnormalities associated with induced pluripotency. The iPSC reprogramming technique has extensive potential for molecular regeneration, disease modeling, and drug discovery.

The somatic cells of elderly donors can be utilized to generate human iPSCs, and cell reprogramming can reverse the key signs of ageing. By lengthening telomeres, reorganizing the mitochondrial network, alleviating oxidative stress, and recovering pluripotency, the reprogramming process transforms aged cells into young condition.[175] iPSCs acquire ESC-like features, especially with

similar mitochondrial properties, and can modulate mitochondrial or oxidative stress pathways leading to a state of rejuvenation.[176] These transformation events can promote an extensive restructuring of mitochondria, including mitochondrial counts, morphology, viability, cellular metabolism, and the complexity of mtDNA. Telomere malfunction and chromosomal fragility can impair the ability of iPSCs to self-renew and the developmental pluripotency to differentiate.[177] Telomere rejuvenation is an aspect of epigenetic reprogramming toward pluripotency and reprogramming can maintain or reverse telomere length and chromatin structure. Moreover, in the cells with telomere and mitochondria defects, somatic cell nuclear transfer (SCNT)-mediated reprogramming might be a better technology than current reprogramming factors.[178] Therefore, the genetic foundation of ageing and rejuvenation can be modeled using iPSC lines, enabling the identification of new factors that prevent premature ageing or impact cell rejuvenation. In addition, iPSCs can be reprogrammed from patient cells via small molecules, miRNAs, and combinations of reprogramming factors, and be differentiated into somatic cells for drug testing and regenerative medicine.[179]

However, many studies clarified that ageing also might constitute critical barriers to cell reprogramming due to cellular senescence, inflammation, telomere reduction, and metabolic alterations.[6] The aged or pathologic tissues are relatively inefficient at reprogramming and iPSCs derived from these tissue types might lack sufficient pluripotency and differentiation ability.[180] Somatic cells produced from iPSCs might undergo premature senescence. The differentiated cells or premature termination of reprogramming can also carry the gene mutation and the full genetic heritage of the patient, which might contribute to long-term risk and tumor formation.[181,182] Thus, the application of iPSCs needs efficient reprogramming and differentiation protocols, and the capability to maintain iPSC functionality in ageing microenvironment. Studies have proved that some longevity-promoting compounds and inhibition of age-related pathways enhance reprogramming in regenerative therapy. There are also many methods to boost the safety of iPSCs, such as using suicide genes to eradicate any undifferentiated iPSCs that remain after therapy, choosing younger donors, using appropriate cell sources, improving gene delivery techniques, replacing DNA delivery with proteins, mRNA, or

regulatory miRNAs, using small-molecule DNA modifiers, and using low-passage iPSCs.[183]

Rejuvenation by lineage reprogramming

Lineage reprogramming, also described as direct reprogramming, is the procedure of switching somatic cells from one lineage to another with no transition for intermediate pluripotent states.[184] This method of cell reprogramming generates particular cell types by ectopically expressing various lineage-specific transcription factors or miRNAs. For instance, recent research demonstrated that certain pro-neural transcription factors can directly reprogram non-neural somatic cells into neurons, skipping the pluripotent stage.[185] Hence, lineage reprogramming techniques can be used to create a variety of cell types, including brain, cardiac, hepatic, and pancreatic cells. Meanwhile, because of the unique advantages of in situ conversion in live organs, lineage reprogramming is efficient and suitable for in vivo tissue repair and rejuvenation. Direct reprogramming in vivo may also benefit from minimizing hazards for genetic changes during prolonged in vitro culture, cancer development associated with de-differentiation, and immunological rejection following transplantation.[116]

The senescent program induced by ageing process and tissue damage can offer a beneficial microenvironment for in vivo lineage reprogramming. Chiche et al. suggested that tissue damage induced senescence and SASP secretion to promote the plasticity of resident cells, promoting in vivo reprogramming in tissue repair and regeneration.[186] However, direct lineage reprogramming might retain epigenetic hallmarks of primary cells, like ageing hallmarks, which makes the reprogrammed cells suitable for modeling ageing-related disease.[187] Thus, assigning a new cellular identity to terminally differentiated somatic cells, lineage reprogramming plays key roles in in vivo repair and rejuvenation. This technology can be built to utilize numerous and convenient autologous patient-derived cell types as a source, and is particularly crucial to replicate age-related traits and mimic the onset pathophysiology of diseases.

Rejuvenation by partial reprogramming

Cell reprogramming is a stepwise protocol. Studies have proved that somatic cell reprogramming mediated by OSKM for fewer than 7 days induced transient cellular alterations and reversible dysplasia, but partial reprogramming induction for more than 7 days could lead to tumor formation.[188] Accordingly, the partially reprogrammed cells via short-term exposure to Yamanaka factors only partially lose their differentiated identity and undergo molecular rejuvenation without dedifferentiating to pluripotency.[189,190] More specifically, partial cell reprogramming indicates that cells gain the ability to multiply and exhibit certain stem cell markers, but do not totally lose the cellular identity or receive all characteristics of pluripotent stem cells.[189] Thus, partially reprogrammed cells have no risk of teratoma after transplantation. The cellular re-differentiation to the original phenotype with epigenome rejuvenation and the ability to react to optimum cocktails of certain differentiation factors is the representative feature of partial reprogramming.[191]

Generally, it is difficult to distinguish between the underlying epigenetic alterations that rejuvenate ageing cells and the changes that regulate the shift in cellular identity. Partial reprogramming is able to restore the common features of cellular ageing without altering the identity or function of the cells.[192] Cyclic in vivo short-term induction of OSKM suppresses age-related phenotypes and histological alterations in different organs. Chondronasiou et al. demonstrated that partial and reversible reprogramming could improve the ageing states in cells, increase the ability of old mice to restore tissue damage, and lengthen the lifespan of progeroid mice.[193] Potentially reversing the effects of ageing or tissue damage through organ-specific partial reprogramming could lead to the regeneration of the desired organ.[194] In addition, partial reprogramming can generate a secretory phenotype that promotes cellular regeneration and improves the chronic inflammatory state linked to ageing and secondary senescence in nearby cells through enhancing SASP.[104] Therefore, partial in vivo reprogramming can improve ageing-related traits, like the diminished capability to combat injury and the loss in the capacity of tissues and organs to regenerate during life.

Epigenetic rejuvenation

Reset of ageing molecular signatures by epigenetic rejuvenation

Epigenetic remodeling is associated with biochemical modifications to the genome, leading to an altered response of gene transcription to physiological stimuli. DNA methylation clocks might detect a wide range of ageing-related epigenetic modifications that are indicative of genomic, cell biological, and tissue changes that occur during life.[195] Epigenetic clocks can be more accurate than chronological clocks at estimating biological age, which aids in predicting human lifetime via age-reprogramming therapies. Protein-protein interactions can induce allosteric regulatory sites in complicated epigenetic machinery.[196] Hence, accessing allosteric sites can assist in the development of epigenetic medicines with improved druggability and pharmacological characteristics. In addition, there are many lifespan-extending conditions, like $Prop1^{df/df}$ dwarfism, calorie restriction, and rapamycin administration, slowing molecular variations linked to the epigenetic clock in mammals.[197] Furthermore, reprogramming aged cells to a more youthful status carries the hazard of tumor formation. During reprogramming without de-differentiation, the mobility of heterochromatin protein 1β, an essential epigenetic modifier, has been proved to increase in SCs and promote epigenetic rejuvenation.[198] The epigenetic rejuvenation with minimal de-differentiation can be realized by OSKM transduction in partial reprogramming. For epigenetic rejuvenation, distinguishing the rejuvenative features of reprogramming from dedifferentiation is a strong development.

Epigenetic regulation of mitochondria during rejuvenation

Mitochondria role in epigenetic processes mostly involves alterations in DNA methylation, histone modification in nuclear chromatin, and posttranslational gene control by noncoding miRNAs.[199] The modulation of mtDNA and mitochondrial proteins by epigenetic and post-translational alterations contributes to the preservation of cellular health and homeostasis. Differential mtDNA methylation is associated with various conditions, including ageing and ageing-related diseases, changed metabolism, alterations in circadian rhythm, and even cancer.[200] Thus, removing or counteracting the effects of mtDNA mutations in mitochondria might extend human health and lifespan.

Moreover, the conserved histone lysine demethylases JMJD-1.2 and JMJD-3.1 could target the H3K27me2/me3 sites, which is also important for UPR^{mt} induction.[201] MET2/ LIN65 histone methyltransferases were proved to mediate the chromatin remodeling and regulate the UPR^{mt}-associated transcriptional networks.[202] These findings revealed an epigenetic mechanism for regulating stress signaling and lifespan in response to mitochondrial abnormalities. Besides, all metabolic intermediates that serve as substrates or cofactors for epigenetic alterations originate from the Krebs cycle and other mitochondrial metabolic pathways.[203] These metabolites contain acetyl-CoA, α-KG, S-adenosyl methionine (SAM), NAD^+, and O-linked β-N-acetylglucosamine (O-GlcNAc) for DNA methylation and histone post-translational modifications, participating in controlling gene transcription and determining cell destiny.

Epigenetic regulation of retro-transposable elements during rejuvenation

Alternate splicing, different promoter or enhancer usage, ncRNAs, and epigenetic changes that impact the structure and function of chromatin all can modulate transcription. Retrotransposon-mediated promoters might also promote gene regulation and expand protein diversity for phenotypic variation and embryo development. During ageing, heterochromatin decay might upregulate the level of silent retrotransposons, leading to promoted mobility of retro-transposable elements (RTEs) within genomes and cellular homeostasis disruption.[204] Chromatin of main retrotransposon classes, such as Alu, SVA, and long interspersed nuclear elements (LINEs), become relatively open in SCs and affect the evolutionarily recent elements, leading to increased transcription and ultimately transposition.[205] Global hypomethylation of the genome can promote genomic instability and RTE activation, contributing to ageing.[206] Hypomethylation of LINEs in cancer cells can restart the recruitment of many variant factors and is connected with an advanced disease stage and poor prognosis.[207] DNA methylation can be oxidized by ten-eleven translocation (TET) enzymes as an aspect of the dynamic demethylation mechanism.[208] TETs are responsible for LINE-1 demethylation in ESCs, but LINE-1s are negatively regulated by further TET-dependent activities. In nascent RNAs of human cells, m^6A actively regulates the expression level of

both autonomous LINEs and co-transcribed LINE relics, facilitating the retrotransposition of LINE.[209]

Epigenetic regulation of inflammation during rejuvenation

The inflammatory response can trigger epigenetic alterations, and epigenetics in turn can interfere with inflammation action. In reaction to severe inflammatory events, transitory activation of NF-κB-related innate immunity and senescence-related inflammatory elements might enhance reparative cellular reprogramming.[210] The expression of numerous pro-inflammatory modulators is regulated by epigenetic procedures, which might therefore play a role in the progression of chronic inflammation. DNA methylation and histone acetylation are correlated with TNF-α expression during development and inflammatory disorders.[211] Combinations of transcription factors maintain the identity of immune cells by controlling the hypo- and hypermethylation of cell-specific DNA. For instance, Sera et al. found that the X-chromosome-specific enzyme, UTX, maintained the expression of downregulated genes during ageing via demethylase-dependent and -independent epigenetic modulation, contributing to hematopoietic homeostasis and inflammation regulation.[212] There are many phytochemicals and short-chain fatty acids regulating DNA methylation and histone modifications, participating in preventing chronic inflammation that worsens neurocognitive and cardiac performance and leads to metabolic disorders.[213]

Restoration of youthful functions in aged cells by epigenetic rejuvenation

Ageing is unavoidably accompanied by a diminished capacity to maintain tissue integrity and function. Cell or tissue rejuvenation without dedifferentiation is known as epigenetic rejuvenation, and it leads to a more youthful functional state and reversed ageing molecular markers.[214] The central epigenetic regulatory mechanisms are based on the enzymes that modulate DNA and histones (methyltransferases, demethylases, acetyltransferases, deacetylases).[17] The epigenome reprogramming can initiate ageing plasticity during heterochronic parabiosis, caloric restriction, or cellular reprogramming.[215] These epigenetic modifications exhibit a strong capability of youthful function restoration in aged cells. In addition, epigenetic modifications can target several druggable pathways. In addition, senotherapy

can increase lifespan, restore the functionality of bone marrow, muscle, and skin progenitor cells, enhance vasomotor function, and decrease the onset of atherosclerosis.[216]

Metabolic manipulation

Mitochondria-based metabolic remodeling

Mitochondria is responsible for the ATP production required for organisms and apoptosis, autophagy, the creation of iron-sulfur clusters, amino acid synthesis, copper and lipid metabolism.[217] The rates of fission and fusion govern the shape, size, and network of the mitochondria, which vary according to both internal and external cues like metabolism and stress.[218] The metabolic condition can affect the form and function of mitochondria, consequently influencing organ function. Conversely, the disturbed mitochondrial dynamics, like genetic ablation of mitochondrial fusion and fission components, also cause metabolic changes. Age-related disruption in energy balance and an increased propensity for age-related illnesses may be caused by the reduction in mitochondrial activity. The crosstalk between mitochondria and other organelles like lysosomes might also lead to increased oxidative stress, reduced ATP production, and breakdown of cellular catabolic mechanisms, ultimately inducing metabolic imbalance and ageing.[219]

The mitochondrial functions in energy homeostasis and metabolism are closely associated with protein quality control factors in disease and age-related disorders, such as PTEN-induced putative kinase 1 (PINK1), Parkin, and TNFR-associated protein 1 (TRAP1).[220] SIRTs control the mitochondrial metabolic checkpoint which can control stem cell maintenance and quiescence, and dysregulation of the checkpoint can deteriorate the function of aged stem cells.[221] Aiming at the mitochondrial metabolic checkpoint might rejuvenate ageing stem cells and improve the functions of ageing tissue. Mitochondria also segregate many critical metabolic pathways, like the TCA cycle, fatty acid β-oxidation, and the one-carbon cycle. The synthesis of mitochondrial metabolites in these pathways might be involved in additional mechanisms that control stem cell activity and fate decisions.[222] Moreover, the mitochondrial UPR^{mt} regulates many genes involved in protein folding, ROS

defenses, metabolism, assembly of iron-sulfur clusters, and modulation of the innate immune response.[223] Independent of the generation and aggregation of ROS, defective mitochondria also play a significant part in the ageing process. The Mitophagy pathway functions as a crucial mitochondrial switching that guides bioenergetic transition and metabolome remodeling attributes, to eventually define the effectiveness and quality of nuclear reprogramming and stemness transition in somatic cells.[224] Mitophagy-induced rejuvenation of mitochondria governs the shift of bioenergetics and metabolome, hence facilitating a change in their capacity for cellular development. Thus, mitochondrial targeting or mitophagy regulation can promote metabolic remodeling, playing potential roles in rejuvenation and regeneration.

Oxidative stress

Deregulation of the redox state causes a rise of peroxides, ROS, and free radicals, which are collectively known as oxidative stress. Adaptive cellular responses to pathogenic challenges in ageing and age-associated disease tolerance, such as ischemia tolerance, can also be greatly benefited by moderate oxidative stress caused by diverse stressors.[225] However, an imbalance between the generation of ROS and cellular antioxidant defenses can result in excessive oxidative stress, which accelerates ageing and the pathogenesis of illnesses like cancer.[226]

It has been demonstrated in numerous human cohorts and animal experiments that oxidative damage and inflammation might promote a state of susceptibility and raise the possibility of unfavorable health outcomes.[227] Studies have suggested that the declined ability in response to oxidative stress with ageing is involved with the activated expression of Nrf2/EpRE signaling and its target antioxidant genes.[228] Brahma-related gene 1 (BRG1) has been proved to protect cells from oxidative stress harm by encouraging the synthesis of antioxidants and inhibiting the generation of ROS.[229] There are also many pathways, including MAPK pathway, PI3K/Akt pathway, heat shock proteins, p53, and NF-κB pathway, playing protective roles in combating oxidative stress for healthful ageing and longevity.[230] Thus,

these pathways might be effective mediators of oxidative stress for metabolic improvement and rejuvenation.

For oxidative damage regulation, bioactive exosomes have antioxidant effects on reducing the excessive ROS, promoting intracellular anti-oxidative stress defense, immunomodulation by blocking excessive ROS and changing mitochondrial function.[231] Antioxidants targeting mitochondria, such as MitoQ and tiron, can penetrate the mitochondrial membrane and neutralize ROS at the core of the origin.[232] Many compounds, like dibenzopyrone phenolic derivatives, caused the nuclear accumulation of Nrf2, stimulated Nrf2-governed cytoprotective gene expressions, and enhanced cellular antioxidant capacity.[233] In addition, interventions including CR and exercise training targeted at restoring endogenous antioxidant ability and cellular stress reaction can contribute to successful vascular ageing and decreased risk for cardiovascular disease.[234] In skeletal muscle, inflammation and oxidative stress are also the primary pathogenic features of ageing, and they are intimately linked to the onset and progression of sarcopenia. There are some promising antioxidant or anti-inflammatory substances, like minerals, vitamins, fatty acids, and antioxidant phytochemicals, to postpone skeletal muscle ageing and the onset of sarcopenia.[235]

Autophagy modulation

Autophagy is a conserved, physiologic, and self-protective mechanism that supports cellular homeostasis and stress adaption. Autophagosomes with bilayered membrane vesicles can capture the degraded cellular components and subsequently merge with the lysosome to digest long-lived proteins, excess or damaged organelles, and misfolded or aggregation-prone proteins.[236] There are three distinct forms of autophagy, namely macroautophagy, microautophagy, and chaperone-mediated autophagy. Depending on the selective autophagic degradation of several organelles, autophagy is subdivided into mitophagy, aggrephagy, pexophagy, reticulophagy, nucleophagy, lysophagy, xenophagy, lipophagy, ferritinophagy, and glycophagy.[237] The autophagy process is important for maintaining cellular energetics, cellular reprogramming, organellar remodeling, immunity regulation, metabolism, and cellular survival.

Autophagy serves as one of the central pathways in the protection against functional loss and increased vulnerability to ageing process and age-related disorders. The activity of autophagy and the autophagy gene transcription by specific transcription factors, epigenetic changes, and microRNAs have emerged as crucially conserved pathways for promoting lifespan.[238] In autophagy pathways, multiple points with age including autophagosome biogenesis, cargo loading, intracellular transport, and autophagosome-lysosome fusion or acidification have shown ageing or disease-associated deficits. Enhancing the function of the autophagy-lysosome system can eradicate age-related organelle degeneration, which might have regenerative benefits for cellular rejuvenation. The regulator of autophagy is the classical mTOR pathway and some mTOR-independent signaling cascades, such as MAPK-ERK1/2, STAT2, AKT/FOXO3, and CXCR4/GPCR, converge into PI3K signaling node.[239] The histone deacetylase SIRT1 also regulates autophagy to influence ageing and age-related disorders.[240] Furthermore, autophagy can eliminate the redundant production of ROS and maintain the cell proliferation capacity and regenerative ability.[241] In addition, autophagy activation in ageing stem cells by genetic and pharmacological methods can improve cell properties and regenerative functions.[242]

Activation of circadian clock

Basic molecular factors controlling circadian rhythms

The molecular circadian oscillator consists of transcriptional and translational feedback loops that are interlocked. BMAL1 and CLOCK (or NPAS2) have the ability to heterodimerize and bind to E-box elements of many clock-controlled genes to drive transcription.[243] Early in the circadian night, PERIOD (PER) and CRYPTOCHROME (CRY) complexes are formed and suppress the transcription that is mediated by BMAL1 and CLOCK. As PER and CRY degrade over the night, negative regulation of BMAL1 and CLOCK is released, allowing the beginning of a new circadian day. These core clock proteins regulate 24 h oscillations in gene expression and activity, and at protein levels. In addition, the molecular clock controls the rhythmic expression of genes related to numerous cellular processes and nutrient-sensing pathways, which provides feedback to the primary clock system.[244] The suprachiasmatic nucleus (SCN) in

mammals serves as the body primary circadian clock, coordinating the timing of rhythmic activity with the light/dark cycle. Almost all the major genes implicated in the modulation of the circadian cycle have been found to induce alterations in circadian rhythms in their absence and to have an impact on health status.[245] With increasing age, the transition toward morningness involves in epigenetics inside the molecular clock, modifications in the master clock, and downstream oscillator sensitivity to SCN signals.[246] Many genes and proteins in circadian expression occur in phase shifts and decreased amplitude.[247] The circadian body temperature, melatonin release, sleep-wake periods, patterns of locomotion, and drinking behavior can all vary with age. Thus, improving intracellular synchronization and the synchronization between SCN network and central and peripheral clocks might restore accuracy and stability to the ageing circadian system. Melatonin, produced in the pineal gland and mitochondria, participates in complicated intracellular signaling pathways with anti-ageing, antioxidant, chemopreventive, immunostimulatory, and tumor-inhibitory functions.[248] SIRT1 modulation is associated with the activity of clock machinery and might resynchronize the dysfunctional cellular key clock circuits.[249] Moreover, many environmental factors, like light exposure, lifestyle, and societal factors, can modify the circadian clock phase.

Circadian clock promotes stem cell rejuvenation

Stem cells contain a functional circadian clock whose rhythmicity contributes to the multipotent cell properties in constant renewal and injury response. Multipotent stem cells from various organs also have distinct clock gene expression profiles with various amplitude ranges.[250] Meanwhile, dephased oscillators can provide stem cells the source of heterogeneity, to respond effectively to varied cues.[251] Numerous embryonic and adult stem cell-dependent activities, including hematopoietic progenitor cell migration, follicular cycle, osteogenesis, regenerative myogenesis, and neurogenesis, have been linked to rhythmic oscillations and circadian clock regulation.[252] The regulation of stem cell division and differentiation by the circadian clock is crucial for adult tissue regeneration. Histone alterations, DNA modifications, non-coding RNAs, huge multisubunit chromatin remodeling complexes, and additional epigenetic changes are also significant points in the circadian modulation of stem cell destiny.[250]

With ageing, stem cells receive signals from endogenous and external factors operated through circadian rhythms and epigenetic clocks. The circadian output and oscillator system have adapted to the particular homeostatic requirements of the adult stem cell region in the young organism. But the circadian functions of ageing stem cells might switch toward a stress-dominated program.[253] Liang et al. found that the overexpression of CLOCK could rejuvenate physiologically and pathologically aged human MSCs.[254] Mechanically, nuclear lamina proteins and KAP1 formed complexes with CLOCK, which preserved heterochromatin architecture and stabilized repeated genomic regions. Hence, circadian clock regulation provides opportunities to rejuvenate stem cells for various tissue engineering approaches.

Tissue-specific circadian clocks influence organ rejuvenation

The oscillations of peripheral clocks in numerous peripheral tissues, including the heart, liver, adipose tissue, retina, and multiple brain regions, can be controlled by synchronizing the circadian clock produced by SCN neurons.[255] Meanwhile, there is a portion of transcripts expressed cyclically in each peripheral tissue, controlling the function of peripheral tissues.[256] These tissue-specific circadian genes govern biological processes necessary for the preservation and dynamic modification of organ functions during the circadian cycle. Cell-autonomous circadian oscillations also have a substantial impact on the physiology and pathology of peripheral organs.[257] In addition, the diurnal pattern of the light-dark cycle has a significant effect on the central SCN clock, while the feeding-fasting rhythm constrains the circadian rhythm of peripheral tissues.[258]

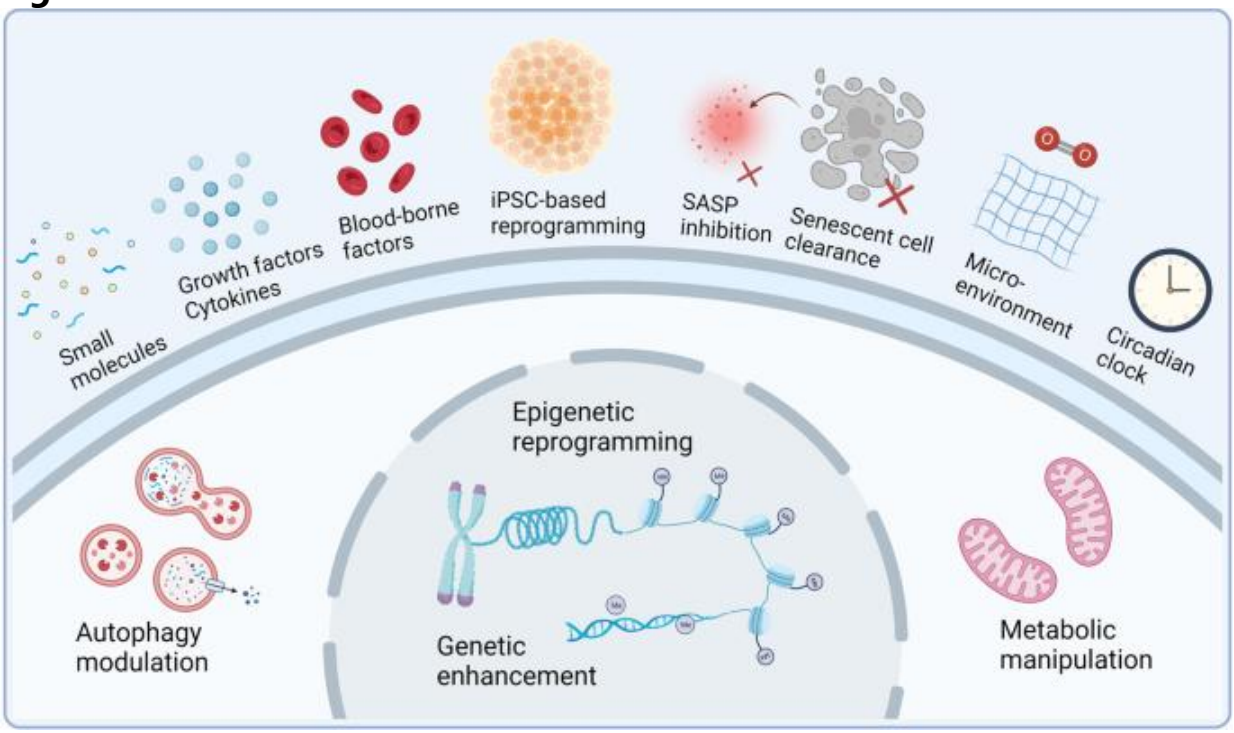
Many studies have suggested that circadian rhythms might be in a noticeably different phase during development in one cell or tissue type compared to another part of the body.[259] Circadian programs are tissue-specific and even varied in the same tissue under distinct physiological states.[260] During ageing, diminished or asynchronous circadian oscillations can alter signaling networks and tissue-specific gene expression profiles. These alterations in tissue-specific clocks might affect immune hyperactivation with ageing.[261] Thus, the tissue-specific targeting of clock subunits by pharmaceutical techniques might aid to

the treatment of age-related diseases and recover circadian coherence with a chronically disrupted clock. Besides, tissue susceptibility and reactions to toxicity also change during the circadian cycle, which indicates the development of drug timing administration.[262]

Designing strategies to promote youthful states in cells and organisms

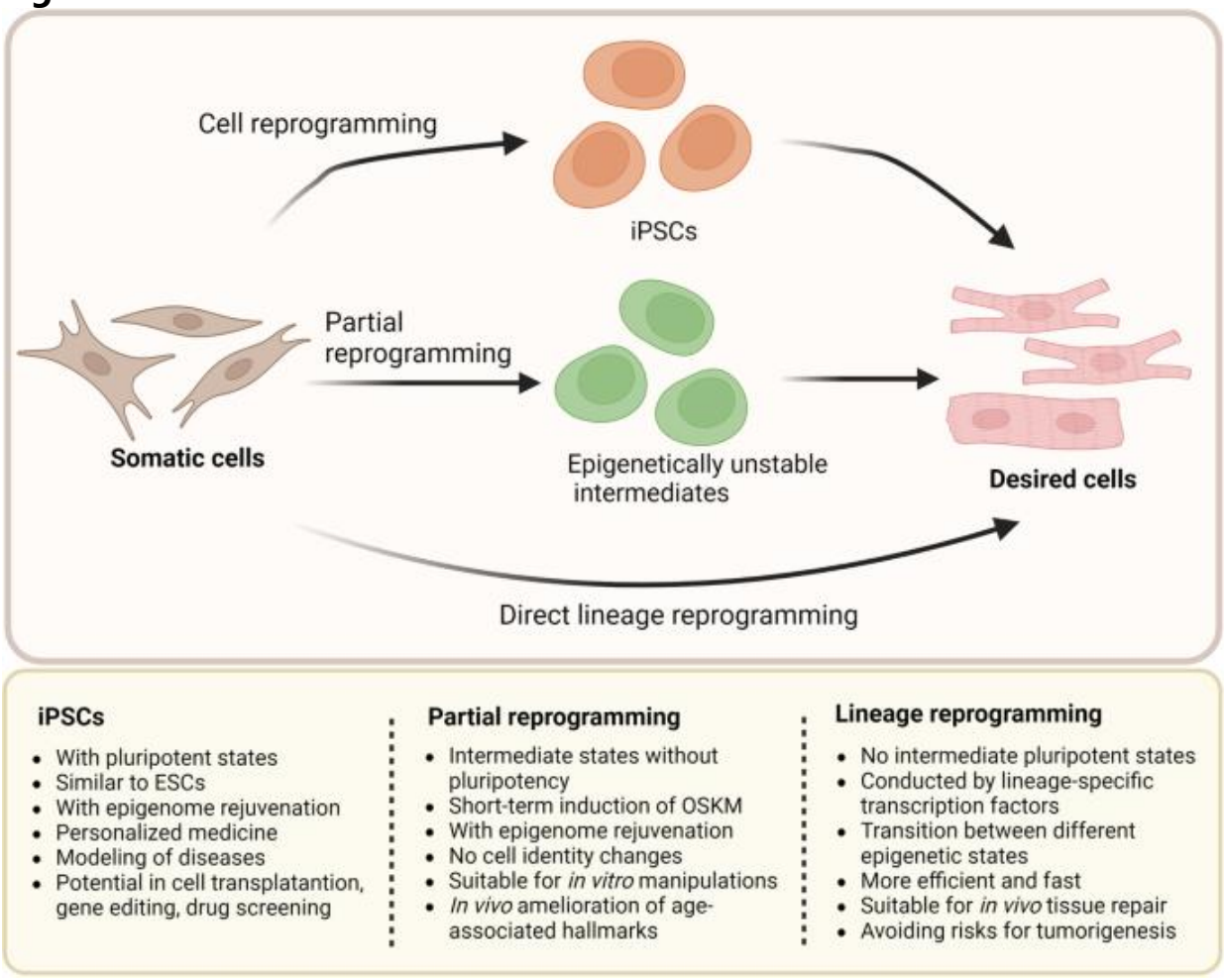
The molecular mechanisms that mediate cellular and organ ageing provide therapeutic targets for cellular rejuvenation. The increasingly rejuvenative approaches have been developed, and the schematic overview of strategies for cell rejuvenation is shown in Fig. 4. It contains cellular reprogramming, clearance of SCs and SASP inhibitor, metabolic manipulation, the restoration of aged stem cell function, microenvironment remodeling, resetting the circadian clock, immune rejuvenation, and heterochronic parabiosis. Especially, cellular reprogramming can rejuvenate the terminally differentiated cells to the pluripotent state or epigenetically unstable intermediates, and also to another desired cell type for tissue repair. The reprogramming technology mainly includes iPSCs, partial reprogramming, and direct reprogramming (Fig. 5).

Fig. 4



Schematic overview of strategies for cell rejuvenation. Various strategies have been developed for cell rejuvenation that leverage intrinsic and extrinsic factors, including epigenetic reprogramming, genetic enhancement, autophagy modulation, and metabolic manipulation. Furthermore, small molecules, growth factors and cytokines, blood factors, iPSC technology, clearance of senescent cells and SASP, microenvironment regulation, and circadian clock modulation can also exert great influences on cell rejuvenation. iPSCs induced pluripotent stem cells, SASP senescence-associated secretory phenotype. Created with BioRender.com

Fig. 5



Reprogramming approaches for rejuvenation. The iPSC-mediated cell reprogramming is a protocol that somatic cells are first dedifferentiated into iPSC and then differentiated into the desired somatic cells. Partial reprogramming refers to a short exposure to Yamanaka factors only generates intermediates with high plasticity. Transforming somatic cells from one lineage to another without transitioning through intermediary pluripotent stages is known as direct lineage reprogramming. iPSCs induced pluripotent stem cells, ESCs embryonic stem cells; Oct3/4, Sox2, Klf4, c-Myc, OSKM. Created with BioRender.com

Resetting epigenetic clock in aged somatic cells by reprogramming strategy

Reset epigenetic clocks by reprogramming to a fully pluripotent state

The biological age of our cells, tissues, and organs is determined by an epigenetic clock based on alterations in the DNA methylation profile. A change in permission for future development and a return to a more flexible and pluripotent state are made possible by the removed DNA methylation instruction, and the production of iPSCs can also get around epigenetic constraints and change DNA methylation patterns to support youthful states in cells.[263] According to the extensive DNA methylation analysis, the aberrant hyper-methylation spread randomly over the genome during the long-term iPSC reprogramming and then gradually diminished. These cells eventually adapted to closely resemble ESCs.[264] Another study that used global DNA methylation and hydroxymethylation analyses demonstrated that DNA demethylation was a miR-29a depletion-mediated process during early reprogramming, and iPSCs produced from miR-29a depletion were epigenetically more similar to ESCs.[265] The reprogramming technology to generate iPSCs has matured greatly. iPSCs can be derived from different tissue-specific cell types, such as human endometrial cells, endothelial cells in the placental artery, amnion-derived cells, fibroblast, blood cells, and even cancer cells.[264,266] In the terms of differentiation degree, terminally differentiated cells and adult stem cells also could be reprogrammed into iPSCs. Senescent and centenarian cells are revived through pluripotent reprogramming, and the iPSCs produced from these cells have reset telomere length, gene expression profiles, oxidative stress, and mitochondrial metabolism.[267] In addition, the fact that these SCs-derived iPSCs can re-differentiate into fully rejuvenated cells further demonstrated that reprogramming is not constrained by cellular senescence and that age-related cellular physiology is reversible.[267] There are a variety of strategies to produce iPSCs, including viruses-mediated gene manipulation (e.g., adenoviral gene delivery), non-integrating episomal plasmids, proteins cell membrane-penetrating, the direct transfection of RNA and chemical reprogramming induced by small molecule compounds.[268] The evolution of iPSC technology is around the improvement of reprogramming efficiencies and the avoidance of gene risk. Currently, given that each random integration event of a retrovirus

poses a possible genetic risk, despite some of these approaches have produced lower efficiency than conventional reprogramming of retroviral administration, they constitute a step closer to therapeutic application.

Reduction in epigenetic age by partial reprogramming

Using partial reprogramming, the epigenetic age of cells can be reduced without losing cell identity, suggesting that full reprogramming of iPSCs is not necessary to reverse ageing of somatic cells. The previous study reported that OSKM + LIN28-mediated partial reprogramming in senescent human fibroblasts could result in the recovery of the high mobility of histone protein 1β, a feature characteristic for young fibroblasts.[198] Likewise, multiple cellular features of dermal fibroblasts from middle age donors were rejuvenated following partial reprogramming with forced expression of OSKM, including transcriptome, epigenomes such as histone methylation and DNA methylation, and functional rejuvenation.[269]

Using a non-integrative partial reprogramming protocol with a cocktail of mRNAs carrying OSKM + LIN28, naturally aged human fibroblasts and endothelial cells exhibited a multifaceted alleviation of cellular ageing, such as resetting the epigenetic clock, reducing inflammatory responses in chondrocytes, and restoring youthful regenerative responses to age, in each case without changing cellular identity.[270] Besides, OSKM-mediated partial reprogramming also restored youthful gene expression in adipocytes, MSCs, and myogenic cells, and the identity trajectory mapped by single-cell genomics revealed the temporary suppression of somatic identity programs.[271] Partial reprogramming emerges as a powerful tool for the restoration of the cell youthful state.

In vivo reprogramming in ameliorating age-related physiological alternations

In vitro cellular reprogramming has observed alleviation of age-associated phenotypes and the reset of age clock. Due to the physiological complexity of the ageing process, it is necessary to study in vivo reprogramming to gain a deeper understanding of how it can affect cellular and organismal ageing. Ocampo et al. reported that transient cyclic induction of OSKM in vivo ameliorated age-associated hallmarks, extended lifespan in progeroid mice,

and promoted tissue repair from streptozotocin-induced pancreatic damage as well as cardiotoxin-caused muscle damage, with no resulting teratoma formation.[272] In the physiological ageing mice, the rejuvenating effects of long-term partial reprogramming are manifested in different tissues, including the skin and kidneys, as well as at the organismal level, and such effects were related to a reversion of the epigenetic clock and metabolic and transcriptomic changes, containing the decreased expression of genes mediating inflammation, senescence, and stress response pathways.[273] Similar studies in naturally aged mice also reported that a single period of OSKM expression can alter epigenetics, transcriptomes, and metabolomes, resulting in a younger configuration in various tissues and in the serum, such as the reversal of DNA methylation changes and transcriptional changes, and the restoration of some ageing-related serum metabolites and biomarkers to young levels.[193] In vivo reprogramming may represent an avenue for facilitating tissue repair. For example, expression of OSKM specifically in hepatocytes can dedifferentiate adult hepatocytes into progenitor cells and promote cell proliferation concurrently, and functionally, short-term in vivo reprogramming increases liver plasticity and promotes regeneration.[274] Heart-specific in vivo reprogramming also induces adult cardiomyocytes to a fetal state, conferring regenerative capacity to adult hearts. and the short-term OSKM expression reduces myocardial infarction-induced damage and improves cardiac function.[274] In vivo reprogramming might also hold a great promise against age-related diseases. In vivo reprogramming with short-term cyclic expression of OSKM also inhibits the progression of intervertebral disc degeneration (IDD), and significantly improves senescence-associated phenotypes in ageing nucleus pulposus cells (NPCs).[275] Exception for OSKM, in vivo cyclic expression of the Forkhead box protein M1 (FOXM1) also ameliorates natural and progeroid ageing phenotypes and increases health span.[276] Despite the great progress of in vivo reprogramming for cellular rejuvenation, there are some problems and limitations facing us. The first is teratomas, a type of tumor that could be cancerous, which may be attributable to too much OSKM induced by inappropriate dosage.[277] Then, in vivo reprogramming is not enough for systemic rejuvenation, because the reprogramming factors fails to be delivered to certain tissues, which also explains why some study switched their focus to local rejuvenation such as NPCs.[275] Finally, several ageing hallmarks such as nuclear and mitochondrial

DNA mutations, metabolic aggregates in the cell and extracellular matrix, cannot be reset through in vivo partial reprogramming.

Epigenetic drugs

Currently, "epigenetic drugs" such as those that modulate enzymes that cause epigenetic changes are being considered as potential treatments against ageing. DNA methylation-targeted drugs mainly contain DNA methyltransferase inhibitors, like 5-Azacytidine. The removal of DNA methylation is a prerequisite for epigenetic rejuvenation and the generation of iPSCs.[265] 5-Azacytidine was used to facilitate the reprogramming of human fibroblast into iPSCs by overcoming epigenetic barriers.[268] Besides, brief methylation inhibition with 5-Azacytidine could confer human dermal fibroblasts a relaxed chromatin structure and short plasticity, and the 5-Azacytidine-treated fibroblasts exhibited the upregulation of pluripotency gene expression and differentiation capacity towards other germ layers.[278] Bromodomain and extra-terminal inhibitors (BETi) that work to repress transcriptional elongation of acetylated chromatin are also for stem cell rejuvenation. BETi has exhibited the effect to induce keratinocytes dedifferentiation, to enhance the migratory ability in keratinocytes in vitro, and to promote skin wound healing in vivo.[279] Then, the high throughput chemical screening discovered I-BET151 can robustly contribute to the expansion of $PDX1^{+}NKX6.1^{+}$ pancreatic progenitors (PPs), and these expandable PPs (ePPs) maintain pancreatic progenitor cell status in the long term and can efficiently differentiate into functional pancreatic β (ePP-β) cells, which optimize the stem cell therapy in the amelioration of diabetes.[280] Histone methylation inhibitors have been reported to re-activate stemness-related genes. The 3-deazaneplanocin A (DZNep) compound, which specifically inhibits H3K27me3 and H4K20me3, is crucial for activating OCT4 during iPSC reprogramming.[281] Another intriguing family of anti-ageing drugs that can significantly contribute to the fight against ageing and age-related disorders are histone deacetylase (HDAC) inhibitors. HDAC inhibitors have the ability to reverse the deacetylation of histone tails and trigger the expression of specific genes.[282]

Rejuvenating strategy by clearance of SCs and decreasing SASP

Elimination of SCs using genetic approaches

Recent advances in our understanding of gene manipulation against age and age-related diseases have prompted several distinct interventional strategies. The first one is the repression of senescence-associated genes to delay or reverse senescence. For example, identifying the driving role of the histone acetyltransferase gene KAT7 in senescence in the Werner syndrome (WS) and Hutchinson-Gilford progeria syndrome (HGPS) models via genome-wide CRISPR-Cas9-based screening. Intravenous administration of lentiviral vectors containing Cas9/sg-KAT7 reduced liver ageing and increased lifespan in progeroid and physiologically old mice.[283] Many genes driving senescence and mediating ageing-related disease have been revealed in the past year, and some resource tools also have been developed to facilitate the identification of genes related to cellular senescence and age, including SeneQuest (available at http://Senequest.net) and GenAge: The Ageing Gene Database (available at https://genomics.senescence.info). These ageing-associated genes provide the therapeutic targets for rejuvenation via gene ablation Then, targeting the inhibition of anti-apoptotic regulators to increase the susceptibility SCs to apoptosis is another approach to eliminating SCs. Analysis of proteomic and transcriptomic datasets revealed senescent cell antiapoptotic pathways (SCAPs), covering Bcl-2 family members, ephrins, PI3K isoforms, p21CIP1, HIF-1α, and plasminogen-activated inhibitors 1 and 2 (PAI-1 and -2), were indeed more highly expressed in senescent than non-senescent cells, which is responsible for the resistance of SCs to apoptosis.[284] The small interfering RNAs (siRNAs) against these anti-apoptotic regulators were effective in killing SCs.[285] However, more than one SCAP pathway mediate the resistance to apoptosis, so the gene ablation targeting a single SCAP pathway may be poor. Furthermore, the dominant SCAPs also vary with different cell types, which expands the limitations of genetic silence of SCAPs to remove SCs. Genetic strategy against senescence also can be achieved by overexpression of longevity gene to reset gene expression in SCs. Telomerase reverse transcriptase (TERT) and follistatin (FST) gene therapy using high-capacity cytomegalovirus vector by intranasal inhalation or injection significantly improved phenotypes associated with healthy ageing and extended lifespan in mice without severe side effects.[286] Specifically, glucose tolerance and physical performance were significantly enhanced.

Further, TERT reduced the telomere shortening associated with ageing, and both therapies halted the degeneration of the mitochondrial structure.[286] A clinical trial about TERT gene therapy with adeno-associated virus (AAV) vector to reverse ageing has been approved, and it is also the first clinical trial of genetic editing against ageing that was registered in Clinicaltrials.gov in the world (NCT04133649). Another study reported the gene therapy with three longevity genes (FGF21, sTGF-βR2, and αKlotho) treated multiple age-related diseases.[287] In obese and diabetic mice, the findings demonstrated that FGF21 alone totally reversed weight gain and type II diabetes, and that when combined with sTGF-R2, renal fibrosis in mice reduced renal atrophy by 75%. Administration of sTGF-βR2 alone or in combination with two other genes improved cardiac function by 58% in mice with heart failure, indicating that the combination of FGF21 and sTGF-βR2 successfully treated all four diseases with improved health and survival. Importantly, the injected gene did not change the animal's native DNA, remained separate from its natural genome at all times, or transmit between living things or to subsequent generations.[287] These results demonstrate the potential of single or combination gene therapy to treat ageing and ageing-related diseases.

Pharmacological intervention targeting SCs

The association between SCs burden and health span helps to discover chemicals that selectively remove senescent cells. The fact that SCs exhibit resistance to apoptosis supports the idea that these SCs rely on antiapoptotic, pro-survival mechanisms to prevent self-destruction.[288] Recent advances have evoked the development of senolytic compounds that work to target the SCAP network nodes and impair the defenses of SCs against apoptosis. Based on bioinformatic analysis, forty-six compounds that target SCAP pathways have been identified as possibly senolytic.[284] Senolytic drugs with established safety profiles or FDA approval for additional human purposes were initially and purposefully chosen for further study in order to speed the transition from the bench to the bedside, such as SRC/tyrosine kinase inhibitor dasatinib (D), natural flavonoids quercetin (Q) and fisetin (F). D has the ability to trigger apoptosis brought on by dependency receptors, like as ephrins, in part through blocking Src kinase.[289] Senolytic flavonoids primarily induce apoptosis by blocking members of the BCL2 family such BCLxL, as well as HIF-1 and

other SCAP network elements.[290] Because the different contributions of nodes across the SCAP network in SCs subpopulations, senolytic drugs have cell or tissue-specific sensitivity. For example, senescent human pre-adipocytes or MSCs are susceptible to D but not Q or F, while senescent HUVECs are susceptible to Q or F but not D.[284] Likewise, targeting a single SCAP may not be effective in getting rid of SCs due to the heterogeneity of SCAPs among various types of senescent cells. Either D or Q alone has the inability to eliminate senescent mesenchymal embryonic fibroblasts from Ercc1$^{-/-}$ mice and bone marrow mesenchymal progenitors from old mice, but the combination of these agents can do that.[284] In vivo experiment revealed that, both in senescent cell-transplanted young mice and naturally aged mice, intermittent oral administration of D + Q alleviated physical dysfunction and reduced mortality hazard to 65%, and meanwhile increased post-treatment survival by 36% while.[291] Senolytic agents that focus on a single SCAP node tend to trigger apoptosis in a restricted range of SCs types. Navitoclax (ABT-263), a potent antagonist of BCL-2 and BCL-xL, eliminates senescent myocytes and hematopoietic stem cells but not senescent pre-adipocyte.[292] As specific BCL-xL inhibitors, A1331852 and A1155463 have senolytic activity against HUVECs and IMR90 cells. albeit not senescent human pre-adipocytes.[290] The discovery of SCAPs promotes the occurrence of more senolytic strategies, and senolytic drugs hold great promise in treating ageing-related diseases.

Immune-mediated SC clearance

Despite the apoptotic SCs induced by senolytic strategies, such apoptotic cells are still finally removed by the immune system. NK cells, macrophages, and cytotoxic T cells are innate and adaptive immune cells that carry out immunosurveillance of SCs and play a crucial role in their elimination when they are young or under physiological conditions. The interaction between the activating NKG2D receptor and its ligands expressed on SCs determines the involvement of NK cells in the elimination of SCs. In cycling human endometrium, decidual senescence is required to support embryonic implantation, and acute decidual senescence can promote the release of IL-15 and attract uterine NKs, further selectively targeting and clearing senescent decidual cells through the interaction of NKG2D and DNAM1 receptors-mediated granule exocytosis.[293] SCs can also be eliminated by tissue-resident

macrophages or circulating monocytes (MOs). After delivery, MOs are crucial in removing senescent uterine cells to preserve postpartum uterine activity, which helps to maintain the likelihood that a second pregnancy would be successful.[294] T cells are also essential players in immune surveillance and healthy longevity. Similar to NKs, CD8 + cytotoxic T cells can function by granular exocytosis and express the same receptor, NKGD2, to identify SCs.[295] By identifying MHC II molecules, $CD4^+$ cytotoxic T lymphocytes can directly destroy senescent tumor cells. Accumulating evidences have demonstrated that SCs could be cleared by immune cells, which could be a lever for lowering the burden of SCs. Future researches need to focus on new approaches, such as the use of chimeric antigen receptors (CAR) or modified T-cell receptors to enhance immune cells' capacity to recognize and eliminate SCs, or explore various approaches including vaccines and small molecule immunomodulators, to enhance the selectivity and efficiency of immune clearance.

Inhibition of SASP

SASP inhibitors, also named senomorphics, target pathways such as p38 MAPK, JAK/STAT pathway, and ILs, further attenuating the SASP of SCs. Suppressing the SASP also has been an alternative strategy for combating cellular senescence-associated phenotypes or diseases. p38MAPK is the MAPK family member and also an important regulator of the SASP, and p38MAPK inhibitors including SB203580, UR-13756, and BIRB 796, potently suppressed SASP expression in SCs.[296] JAK inhibitors repress SASP function, alleviate systemic inflammation, and eventually enhance physical function.[297] Glucocorticoids act as potent inhibitors of selected pro-inflammatory cytokines (IL-6, IL-1α, and MCP-1).[298] As a flavonoid, apigenin (4',5,7,-trihydroxyflavone) could suppress the level of IL-1α, IL-6, and CXCL10, and attenuate inflammation as well as the related chronic diseases of ageing.[299] ROCK inhibitor Y-27632 is also recognized as a senomorphic drug, which has the ability to reduce levels of pro-inflammatory cytokines secreted by senescent normal and dysplastic oral keratinocytes but with no effect on the permanent cell growth arrest.[300] Alternatively, senomorphics may be achieved by specific neutralizing antibodies against individual SASP factors. Of note, unlike the intermittent administration of senolytic drugs, most SASP

inhibitors are needed to maintain suppression of SASP in a manner of continuous treatment, which raises the likelihood of side effects compared to senolytics taken on an intermittent schedule.

Rejuvenating strategies to restore aged stem cell functions

Improving metabolism in aged stem cells

The metabolic disturbance is known to drive the function decline of adult stem cells with respect to ageing, and the mechanism by which nutrient-sensitive signaling pathways such as the mTOR and AMPK pathways play a central role.[301] Targeting nutrient-sensitive signaling pathways to regulate metabolism homeostasis may be the alternative strategy to rejuvenate aged stem cells.

Rapamycin's suppression of mTORC1 slows the ageing of mouse long-term stem cells (LT-HSCs) by maintaining their ability to self-renew and produce blood cells.[302] Rapamycin also rescues adult EpSCs exhaustion and the resulting progressive hair loss in mice.[303] In addition, rapamycin improves MuSCs function through induction of autophagy in the *Ercc1*$^{-/\Delta}$ progeria mouse model.[304] In the *Zmpste24*$^{-/-}$ progeria mouse model, rapamycin-treated primary MuSCs could restore differentiation and proliferation potential and reverse senescence.[305] Despite the well-established effects of rapamycin on lifespan, it remains debated whether this drug is rejuvenating compound. Long-term (1 year) rapamycin treatment increased memory and learning in both young and old mice, but it also improved some of these traits in young animals, suggesting that rapamycin has beneficial effects that are age-independent.[306]

Metformin also contributes to metabolic-induced rejuvenation by regulating nutrient-sensitive signaling.[306] Metformin has been shown to extend longevity and improve health in mice and *C. elegans* by activating AMPK in a LKB1-dependent mechanism.[307] Metformin also prevents the AGEs-induced inflammatory damage and neuronal dysfunction in human neural progenitor cells through activating AMPK.[308] Similarly, metformin rejuvenates ageing oligodendrocyte progenitor cells and restores CNS remyelination capacity by

activating the AMPK-mediated mechanism.[309] Metformin is also shown to directly inhibit mTORC1 for improving the stemness and alleviating senescence of stem cells, such as the reversal of ageing-associated characteristics in intestinal stem cells.[310] Using metformin to improve metabolism for cell rejuvenation and the treatment of age and age-related diseases such as degenerative skeletal diseases is proved to be promising.[311]

Targeting DNA repair pathways to rescue aged stem cell functions

To maintain genomic integrity and tissue homeostasis, adult tissue-specific stem and progenitor cells have defensive mechanisms that reduce endogenous DNA damage. However, the DNA repair response in stem cells declines with ageing, which results in the loss of stem cell properties and DNA damage-caused cellular senescence and organ atrophy.[312] Identifying a suitable target to promote DNA repair may contribute to rescuing the DNA damage-induced function loss in aged stem cells. NAD^+, as hydride-accepting coenzyme, is involved in the DNA repair and preservation of genome stability, and the increased NAD^+ level reduces DNA damage.[313] NAD^+ can act as a substrate for PARP and SIRTs and provide adenylate for DNA ligase IV, facilitating nuclear DNA repair.[313] NAD^+ levels can be increased by supplementation with nicotinamide mononucleotide (NMN), inhibiting NAD^+-consuming enzymes, and regulating NAD^+ biosynthetic enzymes, in which NMN is widely used. In telomere-dysfunctional animals, administration of the NMN preserves telomere length, inhibits the DNA damage response and p53, enhances mitochondrial activity, and reverses liver fibrosis.[314] NMN alleviates retina damage, drops DNA damage by 50%, and eliminates SCs in age-related macular degeneration models.[315] NMN protects skin cells such as keratinocytes from UV radiation-caused DNA damage by enhancing DNA repair, contributing to reverse skin ageing.[316]

Reversing stem cell ageing by improving protein homeostasis

The conducive effect of proteostasis loss on stem cell ageing inspires the strategy of protein homeostasis to protect stem cell function. Chemical/biochemical therapy is an important approach capable of inhibiting the protein aggregation process, and it has received extensive research in “protein misfolding neurodegenerative diseases (PMND)”. Numerous chemical

chaperones, including 4-Phenylbutyric Acid (4-PBA) and its derivatives, have been shown to exhibit anti-amyloidogenic activity and to prevent misfolding protein aggregation. By increasing partially folded proteins and stabilizing their more compact native structures, 4-PBA can reduce the formation of unfolded aggregates and increase the pool of folding intermediates. It can also change the structure of Hsps to increase the exposure of hydrophobic surfaces, which improves Hsp chaperone activities.[317] In the face of misfolding-induced stress, 4-PBA can elevate the proteostasis ability via activating the heat shock response (HSR) and UPR^{mt} pathway.[318] As dual-targeting drugs, chalcone-based derivatives, including nordihydroguaiaretic acid (NDGA), curcumin, resveratrol, quercetin, and isoliquiritigenin, can target both amyloid aggregations and the proteostasis network in vivo.[319] The mechanism is partially linked to Hsps-containing protein complexes and ER stress-mediated cellular activity.

Restoring the stem cell pools in ageing tissues

The depletion of adult stem cell pool with age contributes to tissue degeneration, so maintaining tissue homeostasis into old age requires replenishing the stem cell pools. Stem cell transplantation is a therapeutic intervention, and it has been extensively researched as a way for the replenishment of regenerative cells to provide stem cells from an unaffected donor or gene-corrected autologous cells to the recipient. A large number of adult stem cells are available for autograft or allograft therapy, but only HSCs transplantation is widely accepted and used clinically, which has been demonstrated to be successful in treating hematologic disorders such as leukemia and lymphoma.[320] Animal experiments revealed replenishing the stem cell pools with stem cell transplantation is also possible for other tissue. For example, EpSCs transplantation can regulate inflammation response, remodel the microenvironment of skin wounds, recapitulate tissue integrity and promote diabetic wound healing.[321] MuSCs have the ability to facilitate myofibers regeneration, restore dystrophin expression, and markedly improve contractile function. More importantly, transplanted MuSCs also accessed the satellite cell compartment, replenishing the endogenous stem cell pool and taking part in injury repair.[322] Of note, considering the susceptibility of stem cells to ageing, simple cellular replacement therapy with younger stem cells is

insufficient. It may be worthwhile to pursue methods for reactivating residual stem cells or combining such procedures with stem cell transplantation. For instance, inhibition of the p38 MAPK signaling pathway in endogenous MuSCs can rescue muscle regeneration ability in ageing animals.[323] Triboelectric stimulation boosts the pluripotency and differentiation potential of old BMSCs, enhances their proliferation and reverses senescence phenotypes. The mechanism by which is in a manner of MDM2-dependent p53 degradation.[324] Therefore, triboelectric stimulation can be used to rejuvenate endogenously aged BMSCs and replenish the bone stem cell pool, further resulting in the acceleration of bone repair in elderly patients.[325] In addition to adult stem cells, reprogramming-derived stem cells can act as alternative cell sources for transplants. A recent clinical trial reported implantation of iPSC-derived dopaminergic progenitors into the putamen (left hemisphere followed by the right hemisphere) of a patient with PD indeed improved clinical symptoms of PD after surgery.[326] Reprogramming-derived stem cells are highly amenable to genetic correction and homogeneous than isolated adult stem cells, but there still exist major obstacles limiting the application, including low reprogramming efficacy and potential security concern.

Rejuvenating tissue-specific cells by targeting their microenvironment

Targeting extracellular signals to reverse stem cell ageing

Physiologically, extracellular signaling molecules are cues, such as EVs, neurotransmitters, growth factors, hormones, and cytokines, designed to transmit specific information to target somatic cells or adult stem cells. EVs function as a cutting-edge tool for stem cell rejuvenation because of their ability to transfer genes safely and with systemic effects. For instance, ESC-EVs can directly facilitate pressure ulcer repair in ageing mice through the rejuvenation of tissue-resided senescent endothelial cells.[327] Neonatal MSCs-secreted EVs can deposit PCNA to reverse aged BMSCs and slow age-related degeneration.[328] In addition, neurotransmitters have been reported to regulate the behavior of nerve-dependent tissue stem cells in wound healing.[329] $Lgr6^+$ EpSCs are a regionally restricted population of EpSCs that has the ability to generate all skin lineages and interact with nerves and specialize in wound re-epithelialization, and the activation of $Lgr6^+$ EpSCs rely on

neurotransmitters from neuroendocrine signaling and subsequently, promotes wound repair.[330] These results suggested that targeting the activation of neuroendocrine signaling might be a promising approach to facilitate wound healing.

Rejuvenating aged cells by environmental preconditioning

Injured tissue has a hostile environment, including inflammation, immune impairment, hypoxic stress, and poor blood supply, which degrades stem cell function, promotes cellular senescence, and results in a low survival rate of transplanted stem cells in vivo. Therefore, it is essential for stem cells to remain viable and maintain their potency before inducing a strong repair response. The optimization of the culture environment, such as hypoxic pretreatment, may achieve preconditioning-induced protection for stem cells.[331] The oxygen tensions in natural cell microenvironments appear to be substantially lower. Particularly, ESCs require low oxygen levels to survive, which start at embryonic implantation and last throughout fetal development. The absence of maternal circulation causes a hypoxic environment during embryo implantation, and in the early stages of pregnancy, the oxygen content of the uterine surface is typically only 2%.[332] Placental oxygen levels only rise to about 8% even after the embryo forms the connection to the maternal vascular system.[333] Relative hypoxia is a feature of the normal physiologic environment of ESCs, and thus, low oxygen concentrations enhance the more efficient growth of ESCs. Adult stem cells such as HSCs and BMSCs, similarly live in hypoxic conditions in vivo.[334] For these reasons, hypoxic pretreatment in vitro promotes the long-term expansion of stem cells, delays replicative senescence, and improves the therapeutic potential.

Modulating ECMs for maintaining tissue homeostasis

The extracellular microenvironment's chemical and mechanical characteristics are sensed by tissue-specific cells, which then produce responses that control a variety of cellular processes, such as expansion, migration, and differentiation, and activation, further maintaining tissue homeostasis. For example, integrin receptors-mediated cell adhesion to the ECM is necessary for cell cycle progression, particularly during G2 to M transition and early mitosis.[335] In addition, the structural integrity of the ECM and integrin-

associated proteins may also contribute to cellular energy metabolism, because the extracellular matrix proteins regulate nutrient and hormonal flux to balance the energy uptake.[336] Changes in the biophysical characteristics of tissue affect how mechanical impulses are received by cells in a variety of disease states, inflammatory conditions, and ageing. Through the process of mechano-transduction, these mechanical cues are transformed into biochemical signals that affect immune cell functions such as cell activation, cytokine generation, metabolism, proliferation, and trafficking.[337] As an integral component of all organs, the alternations of biomechanical and biochemical cues exhibit a huge effect on cell behavior and tissue homeostasis. ECM molecules are continuously produced and secreted throughout life, in both physiologically healthy and pathological states, and they regulate a wide range of biological functions, including stem cell differentiation, angiogenesis, innervation, and wound healing.[338] Therefore, the modulation of ECM to induce desirable cell-specific responses for cellular rejuvenation is of great importance to tissue homeostasis. Because ECM has a more favorable pro-remodeling host immune response and can offer a natural, instructional microenvironmental habitat for functional tissue remodeling, ECM-based biomaterials have been emerging and exhibit great variability.

Restoring defective intercellular communications to extend healthy lifespan

Intercellular communication has double-edged–sword activities, contributing to tissue homeostasis maintenance but also detrimental in ageing and related diseases, and altered intercellular communication has been the hallmark of ageing.[7,77] One of the most prominent and important changes in intercellular signaling that occurs with age is “inflammageing”. The dominant role of inflammation in ageing-related intercellular communication raises the potential of anti-inflammatory agents in lifespan. Aspirin is a common example because it can prolong mouse life and promote good ageing in humans.[339] Moreover, age-related changes in the transcriptional landscape of tissues have highlighted the importance of NF-κB-induced inflammatory responses, and the targeted inhibition of this transcription factor may restore the altered intercellular communications for rejuvenation. The conditional expression of an NF-κB inhibitor in transgenic mice’s aged skin rejuvenates the

tissue's phenotype and restores the transcriptional signature associated with youth.[340] The novel relationship between inflammation and ageing is that NF-κB-mediated hypothalamic immunity causes neurons to produce less gonadotropin-releasing hormone (GnRH), and this decrease in GnRH can be a factor in several age-related changes, including decreased neurogenesis, muscle weakness, skin atrophy, and bone fragility. Correspondingly, GnRH treatment rejuvenates ageing-impaired neurogenesis and prevents ageing development.[341]

Restoration of proper circadian clock for anti-ageing

Rejuvenation of the circadian clock by dietary restriction

The use of DR, including time-restricted feeding (TRF) and calorie restriction (CR), has many profound beneficial effects on ageing through the regulation of circadian clock.[342] TRF, also known as limiting the time and length of food availability with no calorie decrease, can trigger clock-associated gene expression to drive rhythms in circadian mutant mice. For example, the transcripts, metabolites, and proteins in WT liver exhibit daily rhythms, whose rhythms are lost in clock gene knockout (CRY1^{-}/CRY2^{-}; PER1^{-}/PER2^{-}; BMAL1^{-}) mice, while TRF partially rescues the disturbed rhythms in these mutant mice.[343] In addition to the liver, TRF modifies the phase and amplitude of the skin's circadian clock to rejuvenate ultraviolet radiation-caused skin ageing in old animals.[344] For lifespan, TRF even drives a clock-dependent increase in autophagy activity to induce longevity extension.[345] CR, a dietary regimen low in energy without malnutrition, can decrease the occurrence of age-related diseases and extends the lifespan via controlling circadian clock. It has been reported that the duration of two months for CR intervention in early life could enhance the amplitude of core clocks in liver.[346] From the regulatory site, the central oscillator is impacted by CR, which also entrains the SCN clock. The SCN clockwork's temporal organization, circadian outputs under the light-dark cycle, and circadian system photic responses are all influenced by CR during the daytime.[347] BMAL1-dependent and BMAL1-independent pathways are the primary transcriptional and post-transcriptional mechanisms by which CR modifies the clocks. A functional circadian clock system is necessary for CR-mediated lifespan extension, as evidenced by the fact that CR fails to extend

the lifespan of BMAL1 knockout mice.[348] Comprehensively, the circadian clock system extends longevity through transmitting the anti-ageing signals brought on by DR.

Genetic regulation of the circadian clock rescues an aged phenotype

Circadian clock-associated genes modulate the extrinsic and intrinsic mechanisms in lifespan modulation and organ ageing. The circadian clock in intervertebral discs (IVDs) is functional and temperature-entrainable, and ageing disrupts the circadian rhythm of IVDs in an inflammation-dependent manner and leads to IDD.[349] Restoring the dampened expression of BMAL1 protects against IDD via inhibiting RhoA/ROCK signals.[350] The expression of CLOCK is increased in OA cartilage, and by reestablishing autophagic flow, ablation of CLOCK increases antioxidant enzyme activity, lowers ROS generation, and alleviates chondrocyte senescence.[120] The downstream gene of CLOCK-BMAL1 complex CRY2 is suppressed in mice with ageing-related OA, which makes the loss of ECM homeostasis in cartilage.[351] It also reported the low mRNA and protein expression level of CRY in the old Drosophila melanogaster. The mRNA oscillatory amplitude of multiple genes involved in the clock mechanism was dramatically increased in elderly flies by restoring CRY using the binary GAL4/UAS system, and the flies with higher CRY also displayed a remarkable extension of health span.[352]

Pharmacological activation of circadian clock for anti-ageing

Numerous parts of human physiology are regulated by the circadian rhythm, opening up windows for interventions that can be made by only giving medications when their targets are at the proper expression level to rescue. There is growing interest in developing small molecules that directly target the circadian system for medicinal benefits. Representative chemicals targeting core clock components include KL001, SR9009/SR9011, and Nobiletin. KL001 specifically interacts with CRY, which prevents ubiquitin-dependent degradation of CRY, leading to extending circadian time.[353] KL001 suppressed glucagon-caused gluconeogenesis in primary hepatocytes and improved glucose tolerance in obese mice.[353] KL001 also extends the lifespan and

modifies the circadian rhythms of Drosophila melanogaster.[354] The metabolic regulators SR9009 and SR9011 pharmacologically target REV-ERB receptors. By lowering fat mass and significantly enhancing dyslipidaemia and hyperglycemia, SR9009/SR9011 can change the circadian pattern of expression of a variety of metabolic genes to enhance energy expenditure and lower obesity.[355] Nobiletin, a natural polymethoxylated flavone targeting agonizing retinoid acid receptor-related orphan receptors (RORs), is a clock amplitude-enhancing small molecule that has the ability to directly engage circadian cellular clocks to support metabolic health in illness models and healthy ageing in naturally ageing mice.[356] Nobiletin is capable of ameliorating steatosis in obesity by restoring aberrant hepatic circadian rhythm,[357] and alleviating cognitive deficits as well as the pathological aspects of AD, such as Aβ pathology, hyperphosphorylation of tau, astrogliosis-associated neuroinflammation, and oxidative stress.[358] There are other small-molecule modulators with the function to regulate circadian rhythm, which do not directly target core clock genes, such as CKI-7 (CK1 inhibitor), Lithium (GSK3β inhibitor), and OPC-21268 (antagonists for arginine vasopressin receptors V1a).[359] The role of these chemical regulators of circadian clock in the rejuvenation of ageing or ageing-related diseases remains being explored.

Organismal rejuvenation via immune system-targeting therapeutic approaches

Therapeutic targeting of immune cells

Immune system is interconnected with all the other systems in the body, and this systemic nature provides the potential opportunity that targeted modifications to a small group of cells (e.g., HSCs or T lymphocytes) for improving the health of various organ systems.

As the origin of blood cell lineages, HSCs are multipotent precursors population that can reconstitute the hematopoietic system and sustain immune homeostasis. Ageing HSCs have less self-renewal activity, fewer cell divisions, decreased homing efficiency and myeloid lineage-biased differentiation as well as reduced output of lymphoid progenitors.[360] HSCs

play a crucial role in the immune system, hence functional restoration of ageing HSCs has great significance to immune rejuvenation and organismal health. HSCs ageing is accompanied by alterations at the gene level, and genetic modulators by ablation of genes driving ageing or overexpression of rejuvenative genes might be strategies to prevent HSCs dysfunction, such as the deletion of the imprinted gene Grb10 or forced expression of Satb1.[90] In addition, pharmacological intervention based on the pathophysiology of HSCs senescence also can promote rejuvenation of the HSC compartment, reconstitute the immune system activity and finally even increase overall lifespan. For example, supplementing elderly mice with a sympathomimetic that specifically targets the adrenoreceptor β3 (β3-AR agonist, BRL37344) greatly improved the in vivo function of aged HSCs.[361] In progeroid mice, BRL37344 can decrease premature myeloid and restore the proximal association of HSCs to megakaryocytes.[362]

Immune lineage-mediated cellular rejuvenation mainly focuses on the restoration of T lymphocytes exhaustion caused by organismal ageing, due to its high susceptibility to ageing-associated changes to the immune system.[363] Adoptive transfer of progenitor T (proT)-cells is the merging approach to facilitate T cells function recovery. Preclinical results have shown that proT cells could effectively engraft involuted ageing thymuses, achieve rapid long-term thymic reconstitution and accelerate T cell recovery.[364] The process is regulated by receptor activator of NF-B (RANK) and receptor activator of NF-B-ligand (RANKL) interactions, which generate chemokine-rich niches within the cortex and medulla that probably encourage the recruitment of bone marrow-derived thymus seeding progenitors.[365] ProT cells are limited to the recipient's major histocompatibility complex (MHC) and produce host-tolerant T lymphocytes after undergoing positive and negative selection in the host thymus. Therefore, ProT cells avoid the clinical concerns brought on by graft-versus-host disease (GVHD), and it provides an alternative cell-based strategy to rejuvenate T-cell immunity clinically. Surprisingly, immunotherapy with T cell activation, such as the blockage of PD-1/PD-L1, provides a novel alternative to rejuvenate ageing phenotypes.[366] In SCs, PD-1/PD-L1 axis inhibition could activate $CD8^+$ T cells and improve senescence surveillance, further resulting in the reduced number of $p16^+$ cells. Besides, anti-PD-1/PD-L1 administration also ameliorated age-associated function decline in vivo,

including decreased SASPs and inflammation, and improved alveolar volume, fat accumulation in the liver, grip strength, and athletic ability.[366] Clinically, immune checkpoint blockade (ICB) has been widely used, and thus, ICB against ageing is promising.

Other immune-targeting strategies

As the key site of T lymphopoiesis, the thymus orchestrates adaptive immune responses, and ageing-associated thymic atrophy or involution contributes to adaptive immune system deviations. Pharmacologic interventions to increase thymopoiesis have been reported, such as IL-21, which exhibits beneficial effects on thymic function recovery and T cell output in the aged models.[367] The effect is systemic but is also transient and limited. Cellular reprogramming provides an alternative avenue for thymus function restoration and immune rejuvenation. Chemical activation of thymus organogenesis program can direct human ESCs to differentiate into thymic precursor lineage, further promoting functional regeneration of human thymus in vivo.[368] First treatment with activin A directs the fate of hESCs towards the definitive endoderm, and the subsequent regulation in signaling pathways of Wnt, TGF-β, Shh, retinoic acid, BMP4 and FGF facilitates the generation of thymic epithelial progenitors (TEPs).[368] hESCs-derived TEPs could yield functional thymic epithelial cells when engrafted into athymic mice. T cells produced in TEPs-recipient mice have functional properties capable of in vitro expansion and in vivo immune responses, and they were detected 10 weeks post-transplantation in the peripheral blood. However, the regenerative efficiency of hESCs-TEPs generated by these protocols is low and there is a progressive decrease in the quantity of T cells produced, which was not sustained over 22 weeks.[368] Thymocyte lineage-specific gene FOXN1 manipulation can drive thymus regeneration and restore its function in the age murine.[369] iPSCs also can be directed towards thymus fate by ectopic expression of FOXN1.[370] FOXN1-mediated iPSC-TEPs contribute to supporting T cell development, and antigen stimulation can cause the produced T cell to activate, which reveals that FOXN1 can promote the functional regeneration of human thymus from iPSCs.[370] Similarly, FOXN1 mediates ectopic thymus regeneration from fibroblasts, and the fibroblasts-derived thymus also can

revitalize the thymic architecture overall, boost the number of naive T cells, and decrease the number of senescent T cells and inflammatory.[371]

Targeting systemic signals for organismal youthful state

Heterochronic transplantation and parabiosis for regeneration

In 2005, heterochronic parabiosis was initially demonstrated to reverse the age-dependent loss in stem cell function by restoring the proliferation and regeneration capacity of aged satellite cells and hepatic progenitor cells in naturally aged mice.[372] Since then, heterochronic parabiosis has been used as an experimental system to examine potential systemic influences on lifespan, age-related diseases, and the process of organismal ageing. For example, in the central nervous system, heterochronic parabiosis showed young mice exposed to an old systemic environment or plasma from old mice exhibited diminished synaptic plasticity, and worse contextual fear conditioning and spatial learning and memory, while systemic infusion of young blood plasma reversed that process.[373] It also revealed that plasma levels of chemokines—including CCL11 were linked to decreased neurogenesis in aged mice. Young mice with elevated peripheral CCL11 chemokine levels in vivo suffered adult neurogenesis loss as well as learning and memory impairments., which provided a new therapeutic target to reverse ageing-caused precipitous decline in neurogenesis.[374] Similarly, experiments using heterochronic parabiosis also identify another circulating factor, named β2-microglobulin (B2M), which damages adult hippocampal regeneration function and cognitive performance in an ageing-dependent manner. Aged mice and young heterochronic parabionts have high levels of B2M in their blood and hippocampus, and the ablation of endogenous B2M can alleviate age-related cognitive loss and improve neurogenesis.[375] In addition, heterochronic parabiosis discovered the potential therapeutic value of GDF11 in age-related cardiac hypertrophy and age-dependent deterioration of the neurogenic niche,[376,377] and the contribution of Ly6G+ plasma extracellular vesicle to improving fracture healing in the elder.[378] Heterochronic parabiosis has been a valuable experimental system for addressing some of the fundamental issues surrounding the systemic regulation of cell and tissue ageing, which provide corresponding therapeutic opportunities for organismal rejuvenation.

Nevertheless, the key obstacles of parabiotic experimentation facing include the difficulty to control experimental procedures, the influences of shared organs, uncontrollable exercise, and an ambiguous blood-sharing onset.

Blood factors to revitalize aged organs and tissues

The persuasive evidence that blood factors affect organismal ageing has been provided by heterochronic blood exchange (HBE), which has shown that circulating factors not only restore youthful traits to aged tissues but also cause systemic senescence in the young organism. Thus, defining the blood mediators of the rejuvenating effects is of great importance to revitalize aged organs and tissues. The deep proteomic analysis from the large-scale population has confirmed many increased plasma proteins with ageing, like sclerostin, pleiotrophin, and transgelin, while epidermal growth factor receptor (ERBB1) and a2-antiplasmin were decreased with age.[379] Quantification of proteomic alternations revealed ageing protein waves across the lifespan, and for example, structural pathways such as the ECM are downregulated at a young age but increased in middle and old age.[379] Metabolomic analysis has identified the blood metabolic profiles that change with ageing or ageing-relevant conditions. A nontargeted, quantitative metabolomics analysis in the blood of 15 young and 15 elderly individuals reported that 14 metabolites showed significantly remarkable age-related alternations.[380] The increasing metabolites comprised citrulline, pantothenate and N6-acetyl-lysine, and were associated with declining renal and liver function, while the decreasing counterparts included NAD^+, nicotinamide adenine dinucleotide phosphate ($NADP^+$) and UDP-acetyl-glucosamine, which mainly were associated with redox homeostasis. A longitudinal examination of the impacts of ageing on the blood plasma metabolome also identified pathways enriched for age-related metabolites embodied tryptophan, nucleotide, and xenobiotic metabolism.[381] HBE also gives clues to the role of circulating EVs in ageing rejuvenation. It was reported that aged mice exposed to young blood showed muscle regeneration, in which serum EVs containing α-Klotho play a key role, and administration of Klotho-enriched EVs into ageing mice could promote muscle regeneration.[382] Specifically, identifying blood factors that promote or antagonize ageing can provide therapeutic opportunities for systemic rejuvenation. For example, dilution of old blood plasma with saline that

contained 5% albumin to create a “neutral” age blood exchange (NBE), which met or exceeded the rejuvenating effects of promoting muscle repair, lowering liver adiposity and fibrosis, reducing neuroinflammation, and boosting hippocampal neurogenesis in elderly mice.[383] These results suggested that blood factors might be the key players in ageing process, and targeting blood factors is expected to yield robust resetting of the systemic signaling to youth.

The importance of cellular rejuvenation in treating human diseases

The accumulation of SCs during ageing accelerates the onset of ageing-related disorders and chronic diseases, including metabolic disorders, degenerative disorders, inflammation, autoimmune diseases, cancers, etc. Deeper insights into the role of cellular senescence in disease pathogenesis highlight the significance of cellular rejuvenation in treating human disorders, and also provides cues for rejuvenation therapies (Table 1). In addition, a variety of pharmacological treatments with the elimination of SCs or reversal of tissue and organ ageing have been developed for cellular rejuvenation (Table 2). The discussion that follows will thus concentrate on the advances in rejuvenation strategies for alleviating human diseases, and the details of clinical trials in this study were shown in Table 3.

Table 1 The cellular rejuvenation used for various disease treatments
Table 2 Applications of pharmacological treatment for cellular rejuvenation
Table 3 Clinical trials of developed therapy in ageing and ageing-related diseases

Metabolic disorders

Diabetes

Diabetes, as a metabolic disorder featured by increased blood glucose, result from defective insulin function, impaired insulin secretion, or both, which has

increased mortality in recent years. The known etiological basis of diabetes can provide hints for rejuvenation strategy for treatment. SCs contribute to the pathophysiologies of diabetes via their direct impact on pancreatic β-cell function, involvement in adipose tissue malfunction, and SASP-associated tissue inflammation.[384] Senolytics for removing SCs and senomorphics capable of attenuating the SASP of SCs can ameliorate diabetes and its complications by alleviating insulin resistance and reducing tissue inflammation.[385] Awaking immune cells to kill SCs is conducive to improving blood glucose regulation function in diabetes treatment.[386] The increasing exhaustion and senescence of β cells with age is also the driver of diabetes, and the reversal of senescent β cells and enhancing its function contribute to the treatment of diabetes, such as novel drugs for antagonizing β cell senescence,[387] regulating metabolism burden to protect β cell function,[388] the inhibition of islet inflammation,[389] in vivo reprogramming for β cell regeneration[390] and epigenetic modifications to rejuvenate senescent β cell, etc.[391] Despite various rejuvenation approaches for diabetes treatment, most are still primarily in a developing, preclinical stage. Currently, systemic interventions for diabetes treatment seem to be more likely from bench to bedside, which have been explored in the diabetes population. Intensive lifestyle therapy at the workplace has been conducted on the diabetes population, and it exhibited great beneficial effects on improving the diabetes symptoms, such as significant weight loss and conducive changes in fat mass, glycemic controlling, and multiple organ insulin sensitivity, in which the underlying biological mechanism contains muscle NAD^+ production, SIRTs pathways, mitochondrial activity and adipose tissue remodeling.[392]

Obesity

Obesity is characterized as an abnormal or excessive accumulation of fat that presents a health risk. The signal transduction pathways mediating obesity pathophysiology have been widely explored. Currently, a variety of anti-obesity drugs targeting obesity-related regulation pathways have been explored and even under clinical trials, such as melanocortin-4 receptor (MC4R) agonists, neuropeptide y receptor y5 (NPY5R) antagonist and glucagon-like peptide 1 receptor (GLP-1R) agonist, etc. The mechanisms of these interventions against obesity are mainly related to the control of calory

intake, glucose balance and thermogenesis.[393] Inflammation and metabolic disturbances associated with obesity could be caused by cellular senescence, and thus elimination of SCs with senolytic interventions may help improve obesity clinically. The previous reported that, in obese mice, the combination of D + Q reduced circulating inflammatory mediators, increased insulin sensitivity, boosted glucose tolerance, and promoted adipogenesis.[394] Metabolic disorders in obese are also attributable to adipocyte senescence and unhealthy adipose tissue remodeling, and likewise, senescent adipocyte ablation alleviates adipose tissue inflammation and improves insulin resistance.[395] All the results suggest that the emerging strategy with the removal of SCs have the potential to address obesity-associated metabolic dysfunction as well as its complication.

Tissue regeneration

Tissue regeneration refers to the partial regeneration of an organism's tissue that has been injured by external stimuli, which resulted from the regulation of tissue-residing cells and tissue microenvironment. Cellular rejuvenation for tissue regeneration is devoted to the enhancement of tissue-specific cell function and the regulation microenvironment. The repair of endogenous tissue-specific stem cells was observed in various damaged tissue, like lens stem (LES) cells for lens regeneration after cataract surgery,[396] activations of $Lgr6^+$ EpSCs to generate all cell lineage of skin,[397] endogenous liver progenitor cell-driven liver regeneration,[398] etc. These encourage the development of exogenous activation of tissue-residing stem cells to recapitulate damaged tissue, such as biomaterials-based delivery system featured by controlled and sustainable drugs or other biologically active substances release, in osteochondral regeneration, wound healing, and spinal cord repair, and so on.[399] The dysfunction and senescence of differentiated tissue-specific cells and microenvironment imbalance are other key players that impact tissue regeneration. For example, SCs-matrix interaction is responsible for the difficulty in healing in chronic wounds, because the SASP and ROS production from SCs results in increased matrix proteolysis and inflammation, dysfunction in stem cell, impaired vascular endothelial cells and exacerbated inflammatory microenvironment, and in turn, the microenvironment disturbance further accelerates cellular senescence.[400] Therefore, the strategies with the

elimination of SCs, improvement of tissue cell function, or restoration of microenvironment homeostasis are promising in tissue regeneration. These approaches contain clearance of SCs with senolytics, exogenous stem cell transplantation for replenishing the stem cell pool, EVs and their engineered derivatives for alleviating cellular senescence, and wound inflammatory and chemical compounds for rejuvenating SCs, etc. However, targeting tissue cells and microenvironment fails to achieve the functional regeneration of the large tissue defect or other severe and irreversible tissue damage, such as deep second-degree skin burns, diabetic ulcers, and massive liver defects. Recent advancements in somatic cell reprogramming technology have brought to light the possibility of functional repair of damaged tissue. Impressively, the regeneration of sweat gland cells by epidermal cell, fibroblasts, and MSCs reprogramming not only promotes wound healing in deep second-degree burned skin, but also confers the sweat function, which achieves the functional repair of damaged skin.[401,402,403] Ex vivo reprogramming has the disadvantages including the dependence on startling cell sources and potential contamination of in vitro manipulation. Currently, to overcome this obstacle, in situ regeneration based on in vivo reprogramming has developed rapidly, which is applicated to the regeneration of pancreatic β cells, induced cardiomyocyte-like cells, expandable neural stem cells, and sensory hair cells.[184] Despite the great leap that has been made, in vivo reprogramming is still in its infancy and lots of bottlenecks need to be overcome.[184] Comprehensively, functional tissue regeneration still remains great challenge facing us.

Degenerative disorders

Bone-degenerative disease

The term “bone-degenerative disease” refers to conditions that progressively deteriorate bones and are primarily defined by degeneration, in which osteoporosis is more prevalent. Clearance of SCs has become lucrative methodology for osteoporosis treatment. Age-related osteoporosis has been demonstrated to be improved by genetic elimination of p16,[404] and a combination of D and Q also performed well in repairing bone microstructure as seen in radiation-related osteoporosis.[405] D + Q for the treatment of

osteoporosis is being tested in the clinical trial (NCT04313634). Senomorphics that prevent the production of pro-inflammatory proteins, like the JAK inhibitor ruxolitinib, significantly reduce age-related osteoporosis by possibly suppressing certain factors like IL6, IL8, and PAI1.[404]

OA is a degenerative disease of the cartilage that develops with ageing and shares a pathogenesis with the SASP and senescence of joint tissue cells. SASP has been linked to cartilage deterioration, and age-related mitochondrial dysfunction and accompanying oxidative stress may cause senescence in joint tissue cells.[406] Senolytic agents and senomorphic therapy have potential therapeutic value and implications in OA treatment. Navitoclax and UBX0101 are potential senolytic agents that work to alleviate OA, and the trial of UBX0101 in OA has been completed clinically (NCT03513016). Fisetin is a more selective senolytic candidate with lower hematological toxicity, which can activate SIRT1 to block the inflammatory response in OA chondrocytes,[407] and Fisetin is now undergoing a clinical trial as a treatment for OA (NCT04210986). Senomorphic agents targeting MMPs can reduce OA symptom, increase type II collagen and inhibit chondrocyte degeneration in WT mice.[408] IL-6 receptor inhibitor tocilizumab is presently being tested in phase III trial for hand OA (NCT02477059).

Neurodegenerative diseases

Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) are three of the neurodegenerative diseases. The accumulating evidences have indicated the contributions of cellular senescence to AD pathophysiology, by which telomerase deficiency and telomere shortening, accumulation of β-amyloid (Aβ), tauopathy, and oxidative stress get involved.[409] Removal of SCs pharmacologically can improve memory of AD by reduction of brain Aβ load and tauopathy. The first-generation senolytic ABT263 is able to clear senescent astrocytes and microglia, modulate tau aggregation and inhibit tau-associated cognitive decline.[410] Likewise, D + Q senolytic treatment reduced Aβ-related oligodendrocyte senescence, improved cognitive impairments, and decreased Aβ load and neuroinflammation in AD mice.[411] In another way, D + Q also decreases cortical Tau-containing neurofibrillary tangles (NFTs) burden, brain atrophy

and neuron damage.[412] However, senolytic therapy is rarely reported in treating PD and ALS. Stem cell-based therapies against neurodegenerative diseases hold promise. MSCs can enhance spatial learning and prevent memory impairment in AD via various mechanisms including reducing Aβ plaques and tau hyperphosphorylation, reversing microglial inflammation, and promoting anti-inflammatory response.[413] Currently, many clinical trials of MSCs in AD treatment have been approved (e.g., NCT03117738, NCT04040348, and NCT02672306). The transplantation of NSCs with paracrine effect can decrease tau and Aβ levels, alleviate neuroinflammation, improve neurogenesis and enhance cognitive function via releasing neuroprotective or immunomodulatory factors.[414] Current stem cell sources that have been implicated in PD include NSCs, human ESCs, iPSCs, as well as directly induced dopamine neurons.[415] In addition, some clinical trials for treating ALS with stem cells were completed and it also gained desired results in various countries, including autologous MSC, endogenous MSC mobilization, NSC (NCT05306457), T-cell vaccination combined with autologous MSC and NSC therapy offers proof-of-concept that stem cell grafting as a therapy for ALS is doable and supports a continuous emphasis on perfecting stem cell-based therapeutic techniques to gain maximum benefit in ALS.[416] Despite certain results of stem cell therapies that have been achieved neurodegenerative diseases, the disadvantages of this approach including the demand for immunosuppression and a neurosurgery operation, the poor maintenance of stem cell properties, and imperfect cell delivery systems, still need to be solved.

Cardiovascular diseases

The incidence and prevalence of a wide range of cardiovascular diseases (CVD) increase as a function of age. Molecular pathways involved in cellular senescence, oxidative stress, insulin signaling, autophagy, and inflammation may link the cardiovascular homeostasis deterioration.[417] Cellular reprogramming is an emerging technology for cardiac regenerative medicine, and it works to treat CVD following three ways: (1) in vitro reprogramming of startling cells such as fibroblasts into cardiomyocytes via initiating cardiac

development program, which provides cell source for cardiac regeneration;[418] (2) in vivo cardiac reprogramming by lineage transcription factors or microRNA manipulation, in which resident cardiac fibroblasts are converted into cardiomyocytes in situ;[419] (3) Partial reprogramming of cardiomyocytes into a fetal state to restore the function of senescent cells via temporarily reactivating the nascent developmental program in mature cardiomyocytes.[420] The management of cardiac inflammation, such as targeting inhibition of IL-1 and inflammasome, also work to alleviate CVD, and oral NLRP3 inflammasome inhibitors and IL-1 signaling antagonist including Rilonacept, Canakinumab, and Anakinra have been under the clinical trials.[421] Senolytics therapy is another potential approach to attenuate or prevent several CVD, which can improve myocardial function and alleviate atherosclerosis by clearance of senescent endothelial cells, senescent foam cells, and senescent T cells.[422]

Chronic lung diseases

Chronic lung disease (CLD) refers to a spectrum of persistent lung conditions that impair quality of life and are typically resistant to treatment. The conventional therapy regime for CLD treatment with nasal medications, immune-suppressive drugs, and surgery (in severe cases) only supports the decrease of disease progression rate. Cellular rejuvenation strategies based on stem cell therapy have ushered an alternative in the treatment of CLD. People with CLD may benefit from stem cells in the following ways: (1) lowering airway inflammation and preventing additional harm; (2) creating new, healthy lung tissue to replace any damaged tissue in the lungs; (3) promoting the development of new capillaries with tiny blood channels to enhance lung function.[423] The candidate cell sources rejuvenating CLD include HSCs, ESCs, MSCs, and bronchoalveolar stem cells), and desired efficacy in stem cell therapies promotes more related clinical trials to be developed. Based on the differentiation potential of stem cell therapy, ex vivo lung bioengineering also offers exciting new therapeutic approaches for CLD, which contains physical stimuli such as air ventilation and blood perfusion and stem cell to create a functional lung organ.[424] In the 3D microenvironment, physical stimuli relevant to lung biology and soluble factors can facilitate the establishment of bioengineered lungs through modulating stem cell fate, which is expected to

be a future alternative for transplantation to treat CLD. However, the most important issues to be resolved are how to provide the lung with the ideal conditions of ventilation, perfusion, and oxygenation during the biofabrication process, as well as the best cell types, media, and growth factors (being likely various for the airway and vascular compartments).

Eye-related diseases

Macular degeneration, often known as age-related macular degeneration (AMD), is a serious eye condition underlined by photoreceptor loss and choriocapillaris and retinal pigment epithelium (RPE) degenerating (CC). The accumulating evidence shows that AMD is mainly attributable to oxidative stress, cellular senescence, and inflammation.[425] The enhancement of autophagy can resist oxidative damage and mitigate the progression of AMD, and there are potential rejuvenative strategies activating autophagy for the alleviation of AMD, including mTOR inhibitors, hormones, melatonin, and antioxidants in the diet, where the interaction between the NFE2L2, PGC-1, p62, AMPK, and PI3K/Akt/mTOR pathways could be extremely important.[425] As a result of the role of cellular senescence in the pathogenesis of AMD, it may be possible to use senolytics that kill senescent cells and stop the bystander effects of SASP to treat AMD. Recently, in a phase I study, Unity Biotechnology Company reports that a single dose of UBX1325 improved wet AMD patients' visual acuity over the course of 24 weeks.

Glaucoma and age-related cataract (ARC) are also chronic, progressive eye diseases, and regeneration and re-functionalization of damaged trabecular meshwork (TM) or lens with stem cells have been regarded as a therapeutic alternative for these two conditions. A number of stem cells, including trabecular meshwork stem cells (TMSCs), ESCs, iPSCs, and MSCs, have demonstrated efficacy in maintaining intraocular pressure (IOP) equilibrium and restoring TM cellularity.[426] These stem cells can home and integrate into the TM tissue, naturally differentiating into functional TM cells as well as secreting factors that facilitate tissue regeneration.[427] For ARC treatment, the preservation of endogenous lens epithelial stem/progenitor cells after the extraction of cataractous lens is a new paradigm, which can achieve functional

lens regeneration and avoid notable risks of complications caused by the implantation of an artificial intraocular lens.[396]

Chronic kidney diseases

Chronic kidney disease (CKD) is charactered by persistent kidney function decline, and its progressive nature often results in end-stage renal disease (ESRD). The growing evidences revealed that cellular senescence, stress-induced premature ageing, SASP, oxidative stress, and inflammation-mediated CKD development.[428] By the selective elimination of SCs, ABT-263 can improve kidney function and stimulate tubular proliferation to reestablish a regenerative phenotype.[429] Subsequently, a clinical trial suggested that senolytics with D + Q improved physical function in CKD patients (NCT02848131). Various drugs targeting SASP, like glucocorticoids, resveratrol and other protease inhibitors have been reported to attenuate CKD, and most of these mainly inhibit NF-κB signals and reduce ROS.[430] Stem cell therapy has become the most likely effective therapeutic method to slow and even reverse CKD progression, in which MSC transplantation is widely explored due to their easy collection, low immunogenicity, and high paracrine potential. Both bone marrow MSC and adipose-derived MSC have been shown to have significant renoprotective effects in CKD, including a decrease in fibrosis and glomerulosclerosis as well as an intrarenal inflammatory infiltrate.[431] The efficacy and safety of using MSC in CKD treatment have been tested in phase I clinical trials, in which two of them using autologous bone marrow MSCs (NCT02166489 and NCT02195323), and others are adipose-derived MSCs (NCT02266394 and NCT01840540). Trials employing MSCs to treat CKD may encounter a number of difficulties, including choosing the best administration method, assuring flourishing, tracking injected cells, immunological rejection, inadequate homing and engraftment, and low immune acceptance.[432]

Fibrosis

The term fibrosis describes the deposition of fibrous connective tissue as a reparative response to injury or damage, particularly during chronic inflammatory disorders, and Fibrosis leads to tissue architecture disruption, organ malfunction, and eventually organ failure. Based on that known molecular mechanism, numerous cellular rejuvenation strategies to treat

fibrosis have been developed. A key contributor to fibrosis is metabolic reprogramming, which includes increased glycolysis, upregulated glutaminolysis, and accelerated fatty acid oxidation.[433,434,435] Targeted inhibition of these metabolic processes can regulate collagen production, reduce ECM accumulation and alleviate progression to fibrosis. Miscommunications of macrophage-fibroblast interactions also result in pathological healing and fibrosis, and the restoration of macrophage and fibroblast crosstalk through IGF1 and platelet-derived growth factor C (PDGFC) can reduce tissue dysfunction.[436] Integrin-mediated TGF-β1 activation is a significant pro-fibrogenic signaling pathway, making it a potentially alluring anti-fibrotic target. Pharmacologically blocking TGF-β1 signaling (e.g., SB431542) or inhibiting integrin signaling (e.g., GSK3008348, PLN-74809, and IDL-2965) have become widely accepted antifibrotic therapy.[437] Various anti-integrin small molecule agents for idiopathic pulmonary fibrosis (IPF) treatment are under the phase 2 clinical trials (NCT03069989, NCT04072315, and NCT03949530). Strong preclinical studies have identified pro-inflammatory cytokines (e.g., IL-13, CCL2, and CCR2/CCR5) as fibrosis triggers, and likewise, the phase 2 trials of IL-13 inhibitors alone (NCT01266135, NCT00987545, NCT00581997, NCT01872689, and NCT01629667) or IL-4/IL-13 antibodies (NCT02921971 and NCT01529853) have been conducted in the treatment of IPF, skin keloids, and systemic sclerosis. In addition, other clinical trials to treat fibrosis through inhibiting inflammation are also explored, including anti-CCR2/CCR5 (NCT02217475, NCT03028740, NCT03059446, and NCT02330549) for liver fibrosis and non-alcoholic steatohepatitis, anti-IL-1 (NCT01538719) for systemic sclerosis, anti-IL-6 (NCT02453256) for scleroderma, and anti-CCL2 (NCT00786201) for pulmonary fibrosis. During the process of fibrosis, myofibroblasts exhibit remarkable inter-lineage plasticity, which provides opportunities for cellular reprogramming in fibrosis treatment. During cutaneous wound healing, neogenic hair follicles-derived BMP signaling can reprogram myofibroblasts into adipocytes.[438] Correspondingly, the BMP pathway activation may be the preventive approach to reduce fibrosis and achieve scarless wound healing. Finally, in vivo reprogramming induces myofibroblasts into other functional desired cells, which can not only attenuate fibrosis and also realize in situ tissue repair.[184]

Inflammation and autoimmune disorders

The strong evidences link chronic inflammation to autoimmune disease pathogenesis, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS), inflammatory bowel disease (IBD), psoriatic arthritis (PsA), primary Sjögren syndrome (PSS) and psoriasis, etc. Inflammatory cytokines (e.g., TNF-α, IL-17, and IL-23), T cell-mediated inflammatory responses, and B cells-mediated antibody production play deleterious roles in the inflammation regulation on autoimmune diseases, which raises the potential for cellular rejuvenation targeted immunotherapy for autoimmune disease.[439] Anti-cytokine therapy has revolutionized autoimmune disorders treatment. Targeting TNF-α therapy including monoclonal antibodies (e.g., infliximab) and one receptor-Fc fusion protein (etanercept) is currently availability for RA, psoriasis, and IBD treatment. Inhibition of IL signaling also has been developed against autoimmune diseases, such as IL-1R antagonist (Anakinra), IL-1R1-IgG Fc (Rilonacept), anti-IL-1β mAb (Canakinumab), anti-IL-6 mAb (Sirukumab, Olokizumab, Clazakizumab, and Siltuximab), anti-IL-6R mAb (Tocilizumab, Sarilumab, and Vobarilizumab), anti-IL-17 mAb (Ixekizumab and Secukinumab), anti-IL-17R mAb (Brodalumab), anti-IL17A/F mAb (Bimekizumab), anti-IL-23/p40 mAb (Ustekinumab) and anti-IL-23/p19 mAb (Guselkumab, Risankizumab, Tildrakizumab, and Mirikizumab). Thereinto, monoclonal antibodies targeting IL-6 (Olokizumab: NCT02760407, NCT02760433, and NCT03120949; Clazakizumab: NCT02015520; Vobarilizumab: NCT02518620) have been registered for RA treatment, and other includes anti-IL-23 mAb (Mirikizumab: NCT03482011, NCT03535194, NCT03556202) and anti-IL17A/F mAb (Bimekizumab: NCT03598790 and NCT03766685) for psoriasis treatment, anti-IL-17 mAb (Secukinumab: NCT04181762) for SLE treatment, and anti-IL-23 mAb (Tildrakizumab: NCT03552276, NCT04314544, NCT04314531, NCT04991116) for PsA treatment. Immunosuppression of T cells activation by CTLA4-IgG1 Fc (Abatacept) and anti-CD40 mAb (Iscalimab) in autoimmune diseases also has been studied. Now, Abatacept (NCT02067910 and NCT02915159) and Iscalimab (NCT03905525) have been tested in phase 2 trials for PSS treatment. B cell depletion therapies show great potential for the treatment of autoimmune diseases, and likewise, a large number of monoclonal antibodies targeting B cells function also has been developed,

such as anti-CD20 mAb (Rituximab, Ocrelizumab, Ofatumumab, and Ublituximab), anti-CD19 mAb (Inebilizumab), anti-BAFF mAb (Belimumab) and anti-BAFF-R mAb (Ianalumab), in which Ublituximab for MS (NCT03277261, NCT03277248, NCT04130997) and Ianalumab for SLE (NCT05126277) are under the phase 2 trials. Treatment and clinical results for people with autoimmune diseases have been changed by improvements in tailored immunotherapy, but immunosuppression based on decreasing inflammation may lead to serious side effects, such as increasing the risk of infection.[440] Therefore, future researches seek to sustain immune defenses while inducing lifelong immunological tolerance.

Other ageing and age-related diseases

Other ageing and age-related diseases are also matters of great concern, including skin ageing, skeletal muscle ageing, reproductive system ageing, and ageing-related hearing loss. Skin ageing is currently a very concerning field, and the reversal or delay of skin ageing has a great market prospect. The process of skin ageing is multifactorial and is influenced by both intrinsic (such as time, genetics, and hormones) and extrinsic (such as UV exposure, and pollution) variables. Senescent skin cells, SASP, oxidative stress, inflammation, and autophagy, all mediate skin ageing pathophysiology. Fu et al. reported the phenomenon that epidermal cells can de-differentiation into stem cells in vivo during wound healing, and this discovery was the first to reveal that adult cells can become adult stem cells, which suggested that the high plasticity of epidermal cells and cellular fate was not one-street.[441] The subsequent work of Fu et al. demonstrated that dedifferentiation-derived cells cultivated in vitro shared phenotypic and functional traits with EpSCs.[442] Much more importantly, Fu et al. further discovered that aged epidermal cells had the ability to dedifferentiate into EpSCs, a process for which activation of the Wnt/-catenin pathway was crucial.[443] Such breakthroughs not only make it possible to reverse skin ageing, but also promote the development of wound repair greatly. Currently, topical cosmetics like sunscreen, retinoids, or resveratrol are the foundation of current strategies for combating skin ageing. However, it is unclear how these products' effectiveness in preventing skin ageing is related. Anti-ageing drugs such as senolytics or senomorphics may be useful for skin rejuvenation, because such drugs have anti-inflammatory

and melanogenic properties that help protect the skin, prevent carcinogenesis, delay ageing, and reduce age-related diseases.[444] Application of antioxidants to reduce and neutralize free radicals is an alternative method to slow skin ageing. The administration of natural products, especially polyphenols with known antioxidative properties, show the ability to enhance or remove the undesirable signs of ageing skin. Evidence-based knowledge suggests that the topical or oral use of various polyphenol-rich plants can prevent or reduce, besides others, undesirable conditions of skin ageing.[445] Enhancing skin cell function and remodeling elastin networks hold promise for the restoration of skin youthful state. The developed strategies include stem cells and related EVs,[446] pharmacological activation of autophagy,[447] maintenance of circadian rhythmicity on skin-resided stem cell[448], and regulation of metabolism homeostasis to restore mitochondrial integrity and metabolic output.[449] Identifying key molecules involved in skin ageing makes great significance to skin rejuvenation. The role of COL17A1 in EpSC competition to orchestrate skin homeostasis and ageing and COL17A1 overexpression against skin ageing also were revealed.[450] Therefore, targeting activation of COL17A1 is the potential angle for skin anti-ageing therapeutic intervention. Y27632 and apocynin have been identified as COL17A1-inducing drugs, which show the capacity to facilitate skin regeneration and reduce skin ageing.[450] Similarly, the miR-31-CLOCK-ERK pathway is a significant contributor to and therapeutic target for skin ageing. By inhibiting this pathway, either through conditional miR-31 ablation or with clinically proven MAPK/ERK inhibitors, one can safely and effectively delay the ageing process of the skin.[451] The molecular mechanisms of skin ageing provide the biological basis for the application of drug-loaded biomaterials to skin rejuvenation. For example, using tetrahedral framework DNA (tFNA) to design a novel bio-switchable miRNA inhibitor delivery system (BiRDS), which can fuse miR inhibitors within the framework and maximize the loading ability. MiR-31 inhibitors-loaded BiRDS have excellent skin penetration and high RNA delivery efficiency, significantly combating skin ageing.[452]

Utilizing rejuvenation for cancer treatment

Cancer and its relation to rejuvenation

While most studies have focused on the function of senescence in non-cancer cells, it is clear that cancer cells may also establish a senescence response, which makes it possible to exploit senescence to treat cancer. Induction of senescence in tumors includes chemotherapy, radiotherapy, cell cycle inhibition, and telomerase inhibition. However, persistently therapy-induced senescence (TIS) may create a pro-inflammatory microenvironment.[453] Meanwhile, SASP can contribute to angiogenesis to facilitate tumor development and an EMT in neighboring cancer cells and induce ECM degradation further resulting in the migration and metastasis of cancer cells.[454] To overcome this obstacle, a sequential therapy regimen consisting of TIS followed by a second compound that specifically kills senescent cancer cells (senolytic agent) is highlighted as a one-two-punch method for treating cancer. For example, navitoclax has shown desired results when combined with various TIS, including ionizing radiation, rituximab (CD20 antibody), doxorubicin, paclitaxel, docetaxel, or gemcitabine.[455] A phase II study (NCT01087151) demonstrated that, in patients with chronic lymphocytic leukemia, rituximab plus navitoclax exhibit higher effectiveness, prolonged progression-free survival, and well tolerance than that of rituximab monotherapy.[456] Other chemotherapeutic agents like etoposide (NCT00878449), irinotecan (NCT01009073), cisplatin (NCT00878449), paclitaxel (NCT00891605), docetaxel (NCT00888108), and gemcitabine (NCT00887757) that are combined with navitoclax also have been tested to treat various cancers. For sequential treatment regimen in cancer, the issue that need to be solved is identifying pro-senescence drugs with high efficacy and selectivity in inducing cancer cell senescence.

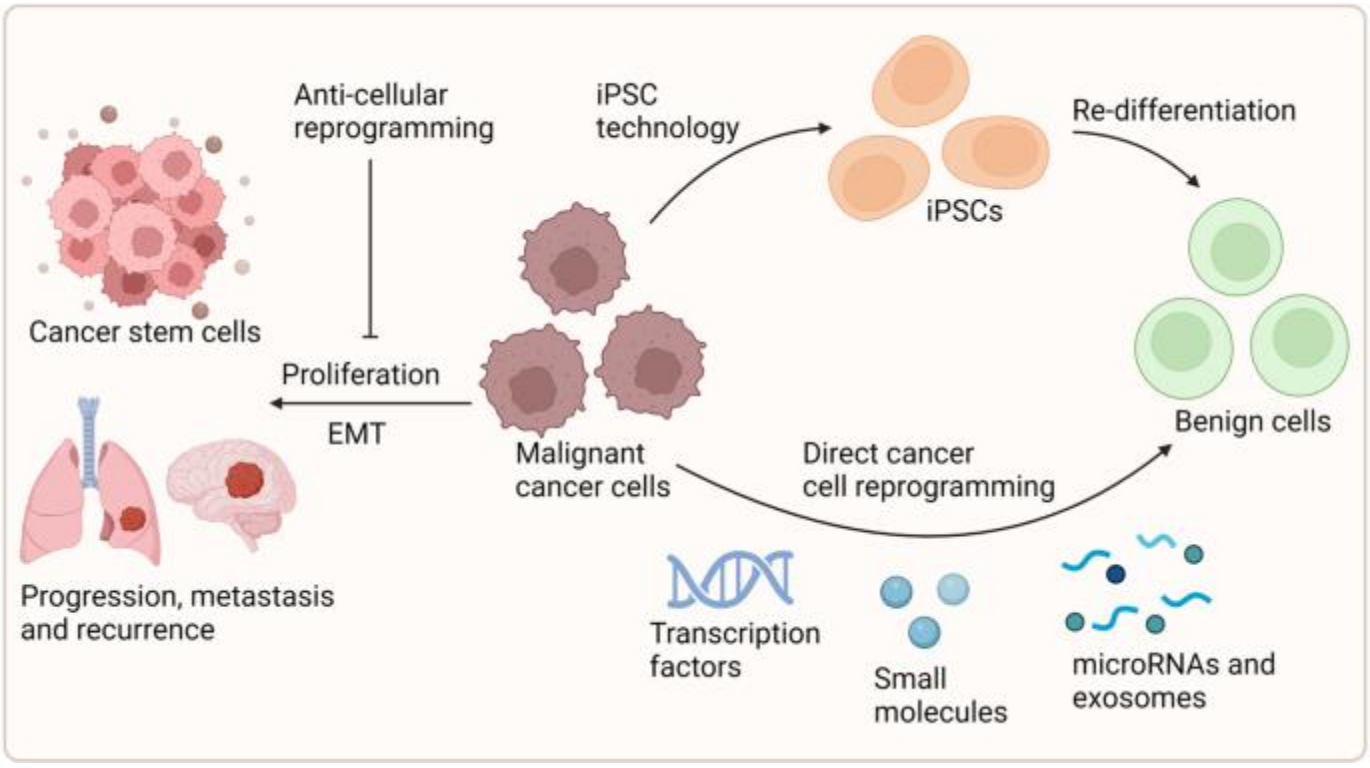
Cellular reprogramming strategy converting malignancy to benignity

It is well recognized that cancer cells can develop a malignant transition from benign cells, but the question that whether it is possible to genetically and epigenetically transform malignant cancer cells to benignity is interesting. The discovery of somatic cell reprogramming encourages the idea that cancer cells, featured by genetic and epigenetic plasticity, can rescue benign cell

functions by cellular reprogramming theoretically. Cell reprogramming techniques that trigger the switch from malignancy to benignity include transcription factors, chemical cocktails, microRNAs, and exosomes (Fig. 6).[457] Transcription factor-mediated cancer cell reprogramming is a pioneering strategy to retrieve benign functions. The conversion of cancer cells to iPSCs with benign phenotypes has been frequently studied. With the induction of pluripotency-associated transcription factors, melanoma cells, hepatoma cells, and colorectal cancer cells, lung adenocarcinoma and gastrointestinal cancer cell can be reprogrammed into iPSCs, and these cancer cells-derived iPSCs maintain benignity without the formation of a visible tumor in vivo.[266] In addition, the cancer cells-derived iPSCs can undergo multi-lineage differentiation, such as epithelial and mesenchymal cells as well as neuron. More importantly, these differentiated cancer cells are less aggressive and lack the ability to form tumors than their parental cancer cells. Lineage-specific factors manipulation also has shown proven capabilities to directly convert cancer cells to desired functional cells without the acquisition of pluripotency. For example, overexpression of the transcription factor CCAAT/enhancer-binding protein alpha (C/EBPα) has been utilized to successfully transform human lymphoma and leukemia B cell lines into macrophage-like cells, with approximately 80% efficiency, and the reprogrammed cells exhibited poor tumorigenicity in vivo.[458] Likewise, hepatocellular carcinoma can be directly induced into hepatocyte-like cells with normal functions when treated with the combinatorial manipulation of hepatocyte transcription factors.[459] Although transcription factor-mediated cellular reprogramming is feasible and ethically acceptable in cancer treatment, such technology has additional challenges including cost and delivery efficiency, and in vivo procedure. The urgent need for developing optional methods is driven by safety and effectiveness concerns brought on by genetic manipulations. The chemical reprogramming technology based on small molecules has some particular advantages, such as affordability, ease of use, versatility that is easily programmable, permeability, and reversibility.[460] Small molecule cocktail (SMC) consisted of SB431542 (TGFβ inhibitor), CHIR99021 (GSK3β inhibitor), BIX01294 (H3K9 methyltransferase/G9a inhibitor), and all-trans retinoic acid (ATRA), could give rise to losing malignant phenotype and acquiring hepatocyte properties in various liver cancer cells.[461] MicroRNA and exosome delivery are novel anti-

cancer alternatives in a manner of cellular reprogramming. Lin et al. reported using microRNA-302s could convert skin cancer cells into iPSCs with decreased tumorigenicity and genomic demethylation. These pluripotent cancer cells displayed more than 86% gene expression similarity to human ESC lines.[462] Under the stimulation of lineage-specific factors, such cells could differentiate into functional cells with benignity properties, like neurons and chondrocytes. ESCs-derived exosomes also deliver ESC-related reprogramming factors to convert malignant cells to benignity ones.[463] However, the precise and efficient regulation of cellular reprogramming to malignancy conversion with microRNA or exosomes faces obstacles, and few investigations have been conducted in the extending frontiers.

Fig. 6



The mechanisms for cellular reprogramming in cancer. Cancer cell reprogramming treatment aims to convert the malignancy to benignity or provide a therapeutic target to inhibit the formation of CSCs. Yamanaka factors-mediated iPSCs technology has been recognized as a common

method for the conversion of cancer cells to benign pluripotent cells, and cancer cells-derived iPSCs can re-differentiate into functional cells with less malignancy and free of tumorigenic potential. Cancer cells also can be directly reprogrammed into benign cells via various reprogramming strategies, such as lineage-specific factors, small molecules, microRNAs, and exosomes. Responsive cellular reprogramming based on EMT contributes to CSCs formation, which mediates the initial, progression, metastasis, and post-treatment recurrence. The role of cellular reprogramming in the formation of CSCs suggests that anti-cellular reprogramming strategies may be considered as a therapeutic alternative in cancer treatment. EMT Epithelial–mesenchymal transition. Created with BioRender.com

Anti-cellular reprogramming therapy for cancer treatment

The increasing evidence suggests that cellular reprogramming also contributes to cancer initiation, development, and recurrence (Fig. 6). Specific transcription factors silence- or epigenetic gene mutation-mediated cellular reprogramming initiate cancer initiation. For example, the depletion of PTEN in NSCs could activate Paired Box 7 (PAX7) and induce the generation of glioblastoma stem cell-like cells, which can lead to intracranial tumors in vivo.[464] Cellular reprogramming mediates cancer development through cancer cell trans-differentiation or dedifferentiation. In non-small cell lung cancer, Lkb1 deficiency induced the trans-differentiation of adenocarcinoma cells into squamous cell carcinoma.[465] In prostate cancer, luminal adenocarcinoma cells may trans-differentiate into neuroendocrine cells as a result of TP53 and PTEN depletion.[466] Cancer cells can be reprogrammed by EMT and cell fusion to acquire stemness and differentiate into cancer stem cells (CSCs), which is a crucial step in cancer development. For example, single-cell analysis of organoids derived from $CD44^+$ colorectal CSCs revealed that TWIST1-mediated EMT played an essential role in inducing cancer cell differentiation, and external stimulation with TGF-β1 can initiate EMT and promote the generation of $CD44^+$ CSCs.[467] Cellular reprogramming generating CSCs also contributes to therapy resistance and the recurrence of cancers, which may be the biological basis of poor clinical outcomes and post-treatment cancer recurrence.

Conventional anti-cancer therapeutics, including chemotherapy and radiotherapy, enable cancer cells to become CSCs. For example, in non-small cell lung cancer, cisplatin-based chemotherapy renders p53 inactive and induces Tribbles Pseudokinase 1 expression, which leads to stemness activation in cancer cells.[468] Therefore, cisplatin plus histone deacetylase inhibitor may synergistically suppress non-small cell lung cancer progression. Anti-angiogenesis therapy also has a great deal of potential in treating cancer, and antiangiogenic agents drive CSCs reprogramming by generating tumor hypoxia microenvironment.[469] CSCs reprogramming also has been observed in immunotherapy, such as adoptive cell transfer (ACT) therapy. In melanomas, T cell-driven inflammatory stimuli lead to melanomas cells with the transition of the differentiated and dedifferentiated state and confer the ACT resistance.[470] Collectively, cellular reprogramming is the key player in cancer initiation, development, recurrence, and therapeutic resistance. Synergistic therapy of conventional therapeutics and anti-cellular reprogramming may represent a promising strategy in cancer treatment. In the future, identifying molecular mechanisms involved in CSCs reprogramming is of great significance to anti-cellular reprogramming therapy.

Summary

The discovery of epidermal cell de-differentiation into stem cells and somatic cell reprogramming into iPSCs revealed that cell fate is not a one-way street. Terminally differentiated cells can reset their genetic and epigenetic properties and acquire the undifferentiated phenotype, and likewise, SCs can also get rid of senescence-related signatures and restore the youthful state. Emerged evidence has indicated the important contribution of cellular senescence in ageing and ageing-related diseases, which encourages the hypothesis that the reversal of ageing may be possible, and targeting cellular senescence may pave the way for that. The development of various cellular rejuvenation strategies provides compelling evidences that the ageing process is not irreversible. Particularly, the effectiveness of stem cell therapy and DR has been tested in the real world, yielding to desired results. Hopefully, there is a great possibility of translation of these rejuvenation strategies to address human ageing, age-associated diseases, and cancers. Therefore, it is reasonable to expect that clinical rejuvenation approaches to treat ageing-

related diseases and even to reverse ageing will be boomed within the next two or three decades.

Conclusions and future perspectives

Throughout human history, the quest to extend lifespan or restore youthful state has been relentless. With the enormous progress in medical technology, including a deeper understanding of cell senescence and age-associated pathophysiology, it has become plausible to extend the human lifespan while preserving health. It is extremely essential to gain insight into the basic principles regulating cellular rejuvenation. Although many aged phenotypic and functional characteristics are studied, the formation, maintenance, and functional contribution to the disease process of aged cells still need further exploration. The study of cellular rejuvenation targeting ageing-related mechanisms is promising to develop potential therapeutic interventions for postponing and reversing ageing, and treating ageing-related diseases.

For decades, one of the dominant theories in ageing research has been that ageing results from the accumulation of DNA changes, mainly genetic mutations, which prevent more and more genes from functioning properly over time. These malfunctions, in turn, can cause cells to lose their properties, leading to the breakdown of tissues and organs and ultimately to ageing and diseases. However, the emerging evidences claim that epigenetic information loss over time is the major cause of mammalian ageing, and epigenetic regulation can restore youthful gene expression patterns. Current advances have provided a comprehensive and detailed multi-level epigenetic panorama of ageing, elucidated some common and unique characteristics of physiological ageing and ageing-related diseases, identified novel biomarkers of ageing, and revealed new mechanisms of epigenetic remodeling in cell and organ ageing. It is expected that future research on ageing epigenetic inheritance will further expand the relationship between chromatin three-dimensional structure and function. Especially, with the development of epigenetic editing technology, scientists can make specific perturbations to the epigenome, to distinguish the causal relationship between the three-dimensional structure of chromatin and cell function. Epigenetic changes caused by ageing in more diverse cell types and pathophysiological states

should also be extensively explored to discover conditional regulation mechanisms of ageing. For epigenetic rejuvenation, developing safe and stable strategies that modulate the epigenetic landscape of aged cells to a primitive state are important for cells to exert rejuvenating effects without cancer risk. Furthermore, systematic comparisons of epigenetic dynamics during ageing and partial reprogramming will contribute to identifying key checkpoints for reversing the ageing process and inform the design of potential intervention strategies for ageing-related diseases.

Pathological accumulation of SCs is also associated with ageing and a range of diseases, and SCs may be potential pharmacological targets for delaying the ageing process. In respect of targeting SCs, there are still many potential markers, like chromatin dynamics and transcriptional signaling, and pharmacological interventions deserving exploitation, to effectively regulate the secretory phenotype of SCs. SC elimination and SASP inhibition have shown some efficacy in clinical studies of treating functional degeneration and chronic diseases in ageing. Notably, SC populations are heterogeneous in terms of composition, function, and tissue distribution, even among species, which is also a problem in the transition from laboratory to clinic. Therefore, identifying the cell or organ-specific markers of SCs is of great guiding significance for future clinical treatment. Targeting the cell microenvironment and systemic signals makes sense for tissue-specific cell and organism rejuvenation. Stem cells play a crucial role in maintaining tissue homeostasis, and cell microenvironments also regulate stem cell behavior, which together form a regenerative unit. External signals from the ageing microenvironment appear to dominate the intrinsic function of young stem cells. In contrast, signals from the young microenvironment may have a limited effect on the regeneration of aged stem cells. It would be interesting to identify the genes or pathways that make aged stem cells insensitive to external signals in young microenvironment. Although many clinical trials registered for stem cell treatments, an effective and safe stem cell therapy to slow or reverse tissue ageing has not yet been identified. Several obstacles still need to be overcome, including proper differentiation and integration of cells in tissues, maintenance of the youth of stem cells and their progeny in ageing tissues, and prevention of tumorigenesis. It will be important to determine the specific mechanisms, which have the potential to provide better treatment pathways-

using stem cell transplantation or utilizing endogenous stem cell banks. Recent advances in single-cell transcriptomics and pedigree tracing techniques provide a systematic understanding of stem cell ageing mechanisms. Developing and utilizing new techniques to track and manipulate stem cells will still be a key field. The systematic identification of gene networks, involved in functional changes, age-dependent changes in RNA and protein and metabolite molecules, and cellular interactions, will contribute to further studies on stem cells in tissue repair and ageing-related diseases. In addition, rejuvenating strong circadian rhythms can optimize organismal physiology and avoid the risk of disease to prolong health. Clinically, circadian rhythms including a series of cyclical life activities, such as diet, sleep and exercise, vary from person to person. It is of great clinical value to develop methods to predict individual circadian rhythms. Machine learning and artificial intelligence methods may help to identify a series of biomarkers to predict circadian rhythms, which has great application prospects for determining the optimal biological clock pattern for each individual.

Despite the great progress in cellular rejuvenation, the potential limitations have led to cellular rejuvenation rarely being tested in human studies. Cellular rejuvenation for reversing ageing and age-related diseases as well as cancers has been extensively studied. While cellular rejuvenation holds great promise, key questions remain to be addressed. (1) Cellular reprogramming strategy can reverse age-related physiological changes and promote tissue regeneration by resetting the epigenetic clock and changing cell fate, but the problems such as relatively low reprogramming efficiency and potential safety concerns, remain the obstacle in the path of its application. (2) The pharmacological delivery system is difficult to express the pluripotency factors with high efficacy. The toxicity might be induced by drug combinations, then reducing the effectiveness of the cocktail and causing side effects in normal cells. (3) Clearance of SCs and decreasing SASP exert a beneficial effect on organ repair and disease treatment, but poor cell selectivity of senolytics may result in the damage of normal tissue, and SASP inhibitors targeting specific secretory factors also have limited therapeutic effects on multiple factors-mediated diseases. Besides, completely senescence-specific markers are still absent. (4) Stem cell therapy as a rejuvenative strategy holds great promise in the reversal of ageing and alleviation of diseases. Despite the advances in

many clinical trials of stem cell therapy, optimizing in vitro culture environment, improving the delivery system of stem cells, and reducing immune rejection are still the major challenges to obtain high-quality stem cells and enhance that therapeutic effect. (5) Restoring defective intercellular communications by the inhibition of inflammation can rejuvenate ageing-impaired changes, but long-term inflammation inhibition may lead to immunosuppression.

Collectively, cellular rejuvenation holds great promise for preventing and treating ageing-related diseases from different dimensions. A healthy and rejuvenated state of the organism can maintain stable characteristics and biological functions without excessive ageing-related degeneration or deterioration. Of note, system therapies such as DR and exercise are available rejuvenation strategies mostly closed to bedside, whose mechanism at the cellular level and molecular level has been expounded.[471] The role of DR in health and diseases has been discussed above. Exercise greatly activates the immune system, facilitates DNA repair processes, maintains metabolism homeostasis, and is capable of lowering the risk of diabetes, obesity, cancer, osteoporosis, AD, and depression as well as prolonging the lifespan.[472] The exploration of diet and exercise programs for different populations is the direction that needs to be paid attention to in the future.

Quantum effects in photosynthetic energy transport

- Hao Tang,
- Xiao-Wen Shang,
- Zi-Yu Shi,
- Tian-Shen He,
- Zhen Feng,
- Tian-Yu Wang,
- Ruoxi Shi,
- Hui-Ming Wang,
- Xi Tan,
- Xiao-Yun Xu,
- Yao Wang,
- Jun Gao,
- M. S. Kim &
- Xian-Min Jin

Quantum Information **volume 10**, Article number: 29 (2024) Cite this article

Abstract

Quantum effects in photosynthetic energy transport in nature, especially for the typical Fenna-Matthews-Olson (FMO) complexes, are extensively studied in quantum biology. Such energy transport processes can be investigated as open quantum systems that blend the quantum coherence and environmental noise, and have been experimentally simulated on a few quantum devices. However, the existing experiments always lack a solid quantum simulation for the FMO energy transport due to their constraints to map a variety of issues in actual FMO complexes that have rich biological meanings. Here we successfully map the full coupling profile of the seven-site FMO structure by comprehensive characterisation and precise control of the evanescent coupling of the three-dimensional waveguide array. By applying a stochastic dynamical modulation on each waveguide, we introduce the base site energy and the dephasing term in coloured noise to faithfully simulate the power

spectral density of the FMO complexes. We show our photonic model well interprets the phenomena including reorganisation energy, vibrational assistance, exciton transfer and energy localisation. We further experimentally demonstrate the existence of an optimal transport efficiency at certain dephasing strength, providing a window to closely investigate environment-assisted quantum transport.

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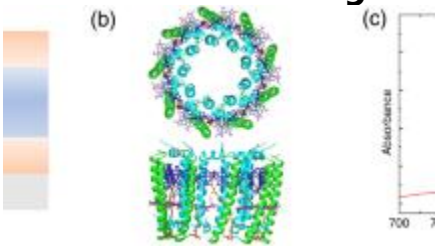

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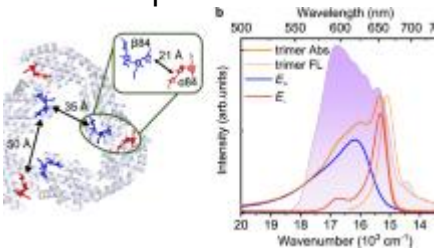

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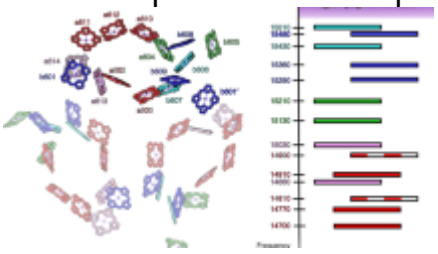

Vibronic mixing enables ultrafast energy flow in light-harvesting complex II

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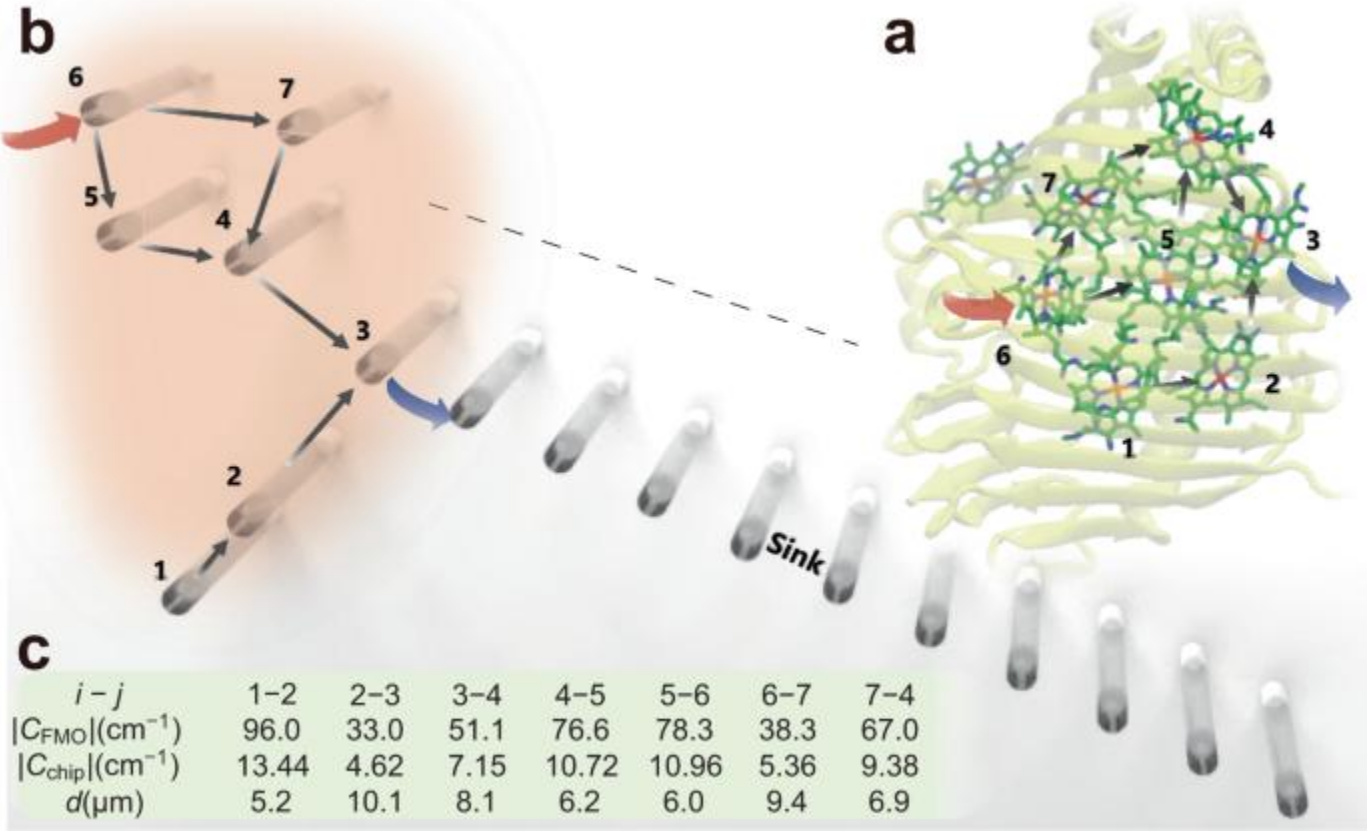
Introduction

Since experimental evidences for quantum coherent energy transport have been successively observed in many pigment-protein complexes[1,2,3,4], the photosynthetic light-harvesting process began to be investigated as open quantum systems that blend the quantum coherence and environment noise[5,6,7,8,9,10]. The theory on environment-assisted quantum transport (ENAQT)[11] was then raised to suggest the enhancement of energy transport efficiencies by environment noise in many nanoscale transport systems. The ENAQT theory has been extensively studied and showed good interpretability on energy transfer among many coherent and incoherent theories[12,13]. The ENAQT theory has now been applied to a rich range of research areas including light-harvesting phenomena in nature, the solar cell engineering and other novel biotic excitonic devices[14].

One of the most well-studied natural creatures for its light-harvesting process is the green-sulphur bacteria, since its structure is simple but highly effective to allow for enough harvest of energy from the very dark deep sea environment[15]. The bacteria collect light through their large chlorosome antenna and transfer excitons to their reaction center. The cable connecting these two part is the so-called Fenna-Matthews-Olson (FMO) complex[16], which is normally formed in a trimer of three complexes with each one consisting of eight bacteriochlorophyll-a (BChl-a) molecules[10]. Seven of the eight molecules are bound within a protein scaffold, which forms the environment for the complexes and provides the source of noise and decoherence. The eighth BChl outside the protein scaffold assists the transport of the excitation into the seven-site structure[17], where the seven BChls are conventionally numbered from No. 1 to 7 (Fig. 1a). The seven-site FMO complex is a prevalent structure for the exciton transfer process. The excitation energy normally transports from BChl 1 or BChl 6 all the way to BChl 3, and eventually goes to the reaction center to accomplish the energy conversion reactions for photosynthesis.

Fig. 1: Experimental layout.



$i-j$	1–2	2–3	3–4	4–5	5–6	6–7	7–4
$\|C_{FMO}\|(cm^{-1})$	96.0	33.0	51.1	76.6	78.3	38.3	67.0
$\|C_{chip}\|(cm^{-1})$	13.44	4.62	7.15	10.72	10.96	5.36	9.38
d(μm)	5.2	10.1	8.1	6.2	6.0	9.4	6.9

Schematic diagram of (**a**) the FMO complex (Protein Data Bank accession 3ENI, image of 3BSD[58] created with VMD[59]) and (**b**) the three-dimensional photonic waveguide array simulating the FMO complex. The numbers of the 7 sites in the FMO complex and their corresponding waveguide are marked. The arrows show that the energy comes into the FMO complex or the waveguide array from Site 6 and moves through Site 3 to the sinks. **c** The absolute values of the coupling coefficient in the FMO complex of *C. tepidum*, C_{FMO} (unit: cm^{-1}), according to[40,41]. In order to map C_{FMO} onto the photonic lattice of a suitable propagation length, C_{chip} (unit: cm^{-1}), all the coupling coefficients on chip, are proportionally reduced to 14% of C_{FMO}, which only affects the overall evolution time, but not the coupling profile among the seven sites. d (unit: μm) is the center-to-center waveguide spacing between two waveguides. Such d values are set to generate the expected C_{chip} values above, as C_{chip} exponentially decays with d, which has been fitted by: $C = 47.19 \times e^{-0.2243d}$. The coupling coefficients between other sites are much weaker (below 15 cm^{-1}), causing very marginal influences on the evolution pattern, and hence are not shown in the table.

Theoretical quantum physicists have investigated the FMO structure[6,7,8,9,10] and proven that there exists certain optimal environmental noise levels to assist for an optimal energy transport efficiency. In recent years, many experimental simulations for ENQAT, especially in the context of a photosynthetic complex model, have emerged[18,19,20,21,22,23]. They are implemented in different systems, including a programmable nanophotonic processor with discrete-time evolution[19], superconducting circuits[20], the nuclear magnetic resonance[21], and the ion-trap qubits[23], with a key goal on introducing controllable environmental noise into the original quantum system[24]. However, there lacks a solid mapping to the FMO photosynthetic energy transport due to various constraints. Firstly, the quantum simulator hardware did not load the full Hamiltonian matrix for the authentic FMO in nature, since simulating the coupling profile for the seven BChls demands strong capabilities on setting the two-dimensional coupling space and flexibly tuning the coupling strength. Secondly, the issue on noise for FMO was not addressed. Some studies simply use white noise[18,19,20], while some analyze coloured noise[21,23], which still does not match a spectral density for real FMO. Thirdly, there are many up-to-date works on photosynthetic energy transport[25,26,27]. Many important issues like the reorganisation energy dynamics and the assistance by vibrational coherence[28,29,30,31,32] are left for further investigations.

In this work, we present a close investigation on simulating photosynthetic energy transport in our three-dimensional photonic lattice. We map the full coupling profile of the seven-site FMO structure on a physical quantum simulator. Besides, by implementing the $\Delta\beta$ photonic model[33,34,35,36], we introduce independently controllable noise for each waveguide, which allows us to load the site energy and the coloured noise that yields a spectral density consistent with that for the actual FMO complex. We demonstrate the photonic model can simulate important biological issues including the reorganisation energy and the vibrational assistance. Furthermore, by mapping the Hamiltonian matrix and coloured noise for FMO on our photonic lattice, we carry out a quantum simulation experiment and demonstrate that an optimal energy transport efficiency exists at a certain noise amplitude. Our work provides a window to closely investigate environment-assisted quantum transport, and may inspire further explorations on comprehensive mechanisms for light-harvesting photosynthesis.

Results

Three-dimensional photonic array provides a highly versatile platform for quantum simulation. The longitudinal direction corresponds to the evolution time and the cross-section of the array structure could be engineered to implement a designed Hamiltonian matrix. Photons propagating through an array of N coupled waveguides can be described by a $N \times N$ Hamiltonian matrix:

$$H=\sum iN\beta iai\dagger ai+\sum j\neq iNCi,j(ai\dagger aj+aj\dagger ai), \quad (1)$$

where the diagonal values of H are β_i, the propagating constant along the ith waveguide, and the off-diagonal terms are $C_{i,j}$, the coupling coefficient between waveguide i and j.

We use seven waveguides to represent the seven sites of the FMO complex (See Fig. 1a, b). Since the coupling coefficients between two adjacent waveguides, C_{chip}, are characterised to follow an exponential decay with the center-to-center waveguide spacing d[37,38,39], we are able to quantitatively control C_{chip} by carefully designing the waveguide configuration (See Fig. 1c). Therefore, utilizing the two-dimensional evolution space, we faithfully map the major coupling coefficients for the real seven-site FMO complex of *C. tepidum*[40,41] on chip. The full information on the Hamiltonian matrix for the FMO molecule is given in Supplementary Note 1. An extra array of 100 waveguides is connected to Waveguide 3 to serve as the sink, resembling the light transport from Site 3 of the FMO complex to its reaction center (Fig. 1b).

Δβ photonic approach

Our photonic array is essentially a quantum evolution system for pure quantum walks, if all propagation constants β_is in Eq. (1) remain constant in time. Here we manage to modulate the diagonal term of the Hamiltonian by introducing $\Delta\beta$, the detunings of the propagation constant β, in order to create a fluctuation of the site energy[33,34,35,36] (Fig. 2a). A large number of stochastic $\Delta\beta$ detunings constitute the quantum stochastic walk[36] and faithfully implement the dephasing process in the open quantum systems[11,34]. The introduction of $\Delta\beta$ can be experimentally achieved by tuning the laser writing speed during the waveguide fabrication process (see details in Supplementary Note 2). The existence of $\Delta\beta$ also causes some fluctuations of the effective coupling coefficient denoted as ΔC, but the value is minor and it is shown to have very marginal influence on the transport efficiency. We hence mainly consider the model with only diagonal $\Delta\beta$ terms (see discussions on ΔC in Supplementary Note 3).

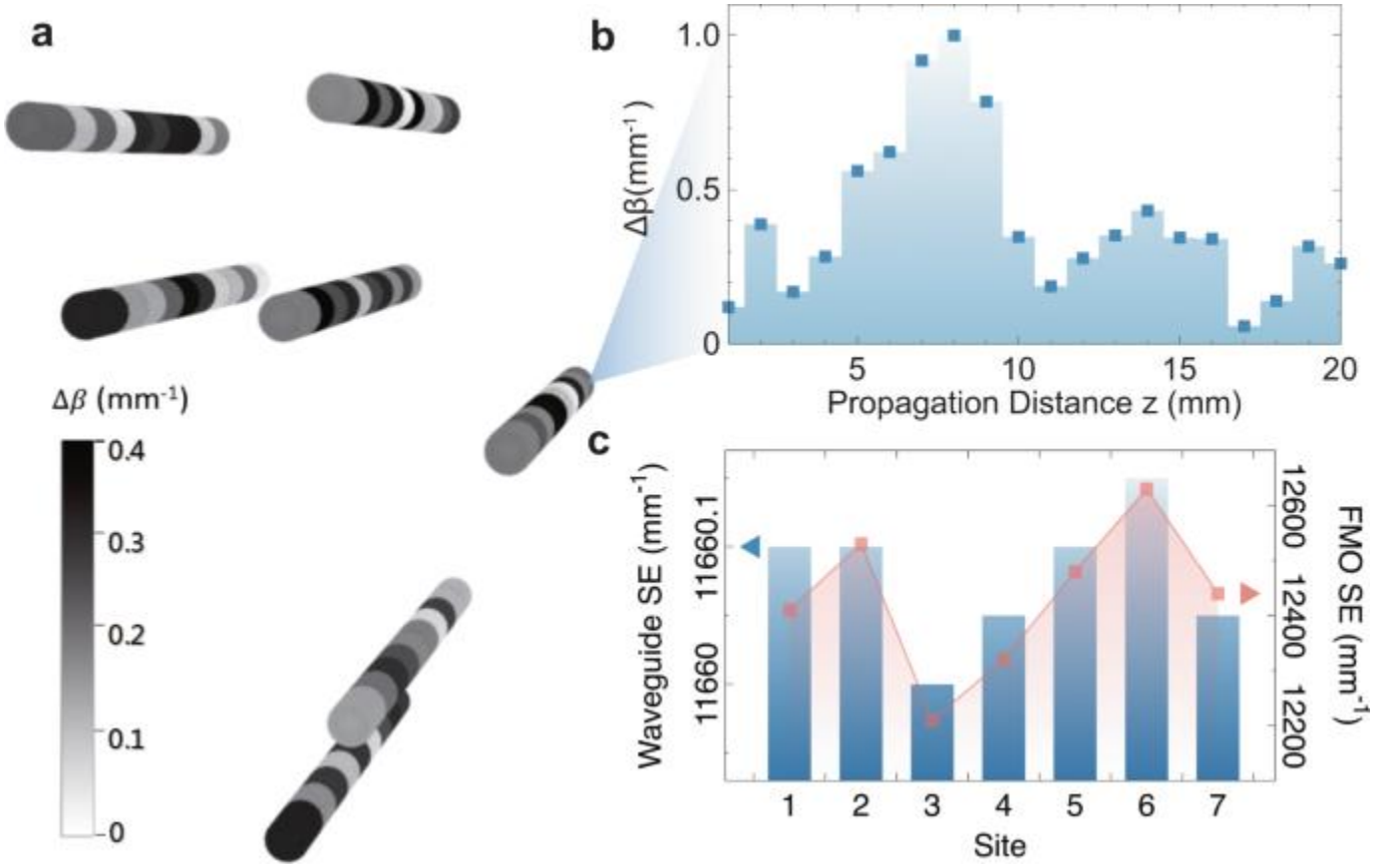


Fig. 2: The $\Delta\beta$ photonic model.

a Schematic diagram of the randomly varying propagation constants shown in different grayscales along the propagation direction of the seven-site structure. This set of random values is one example of the cases with a $\Delta\beta_A$ of 0.4 mm^{-1}. **b** The arrangement for the coloured noise by the $\Delta\beta$ detuning for one site. **c** The site energies (SE) for the seven sites of FMO and the base $\Delta\beta$ detuning used for the seven waveguides to match the site energies.

Using this photonic model, consider the case where each waveguide is broken up into many segments. We then have an effective piecewise dependent Hamiltonian:

$$Heff(tn)=\sum iN(\beta i0+\Delta\beta i(tn))ai\dagger ai+\sum j\neq iNCi,j(ai\dagger aj+aj\dagger ai), \quad (2)$$

where β_{i0} is the base propagation constant for waveguide i, and $\Delta\beta_i(t_n)$ is the extra detunings at segment t_n. Then we have the wavefunction: $\Psi(tn)=e-iHeff(tn)\Delta t\Psi(tn-1)$, where Δt is the time interval for segment t_n.
Such an effective Hamiltonian H_{eff} can be straightforwardly mapped to the real modulation in segments of the photonic lattice. As shown in Fig. 2a, we set the waveguide into segments of equal length with Δt to be 1 mm, and we introduce various random $\Delta\beta$ values ranging between 0 and a given amplitude denoted by $\Delta\beta_A$. At the end of the waveguide, measuring the light intensity distribution gives $|\Psi|^2$.

In this experiment-friendly photonic model, we are able to introduce the noise consistent with the actual FMO complex. Instead of plain white noise, the biological energy transport involves coloured noise that exhibits a non-Markovian nature[31]. Fig. 2b shows an example on the $\Delta\beta$ values at Waveguide 7 that yield coloured noise for BChl 7 (see details on generating white and coloured noise in Supplementary Note 4 and 5). Besides, the base site energy for the seven BChls in FMO varies. In order to reflect that, we additionally consider different base values of β_i for the seven waveguides representing the seven sites, as shown in Fig. 2c.

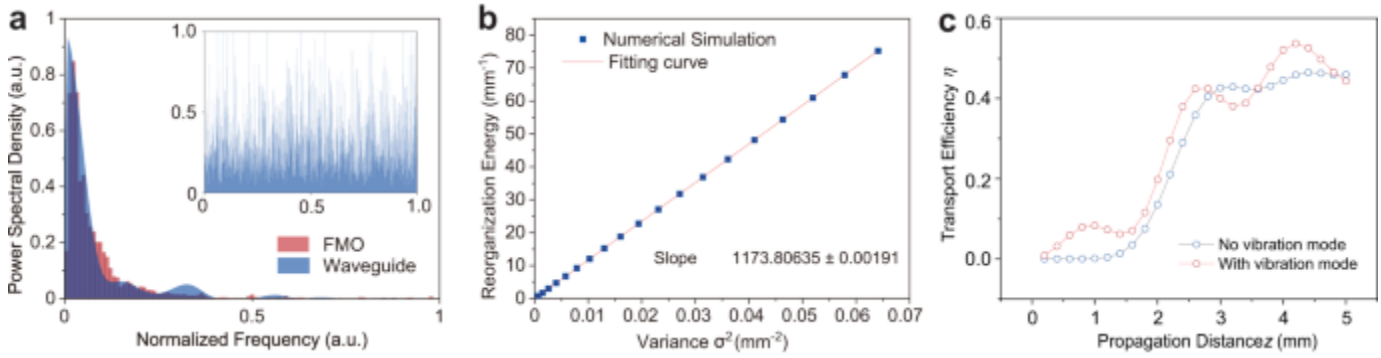
Power spectral density

According to Wiener–Khinchin theorem, the power spectral density $J_i(\omega)$ is the Fourier transform of the original correlation function of the signal. In the context of the $\Delta\beta$ photonic model, it is

$$Ji(\omega)=\int_{-\infty}^{+\infty}d\tau e^{-i\omega\tau}\langle\Delta\beta i(\tau)\Delta\beta i(0)\rangle. \quad (3)$$

In Fig. 3a, the red bars show the pattern of the intermolecular spectral density for BChl 7 of the actual FMO complex[29,30]. The spectral density is obtained via normal mode analysis of the whole pigment-protein complex with the charge density coupling method for the local optical transition energies of the pigments by Klinger et al.[30]. It shows that the noise power for actual FMO complexes is concentrated in the low-frequency components, with some fluctuations in the mid-frequency region, and converges to 0 in the high-frequency region. Note that a hypothesis on site-independent spectral density is adopted for simplicity[25], and the distributions of power spectral density at the other six sites are in similar patterns[30].

Fig. 3: Photosynthetic energy transport using the Δβ photonic model.



a The spectral density formed by the coloured noise introduced on Waveguide 3 is shown in the blue shadow. The red bars show the power spectral density for BChl 3 in a pigments of the monomeric subunits of the FMO protein via normal mode analysis from Ref. 30. Inset shows a spectral density generated from the white noise. **b** Reorganisation energy versus noise variance at Site 7. The sampling frequency and period are f_s = 1 mm^{-1} and t_c = 20 mm,

respectively. The variance $\sigma 2(\Delta\beta 7)=\langle(\Delta\beta 7)2\rangle-\langle\Delta\beta 7\rangle 2$ dependents on $\Delta\beta_7$ amplitude $\Delta\beta_{A,7}$. The parameters of the linear fitting are given in the bottom right of the figure. **c** The transport efficiency at an early transport length with (red) and without (blue) vibrational assistance. $\Delta\beta_A$ for all waveguides are set to be 0.5 mm^{-1}.

The blue shading area in Fig. 3a represents the power spectral density for Waveguide 7 we generate using the coloured noise via $\Delta\beta$ detunings. It matches the normalized pattern for actual FMO complex in terms of the shape on concentrating in low-frequency components. On the other hand, in the inset of Fig. 3a we show a distinct pattern generated from the white noise. The white noise is actually Markovian while the coloured noise exhibits strong non-Markovianity that does exist in the FMO complex[31,42]. See details on noise characteristics in Supplementary Note 6.

Reorganisation energy

The photoexcitation is always accompanied by another energy transfer process. After the FMO complex is photoexcited to a localized excited state, the nuclei inside will undergo a relaxation process to achieve a new equilibrium position. The energy released during relaxation is characterised by the reorganisation energy E^R[25,27], which generally indicates the strength of system-bath coupling[27]. E^R for each single site i can be calculated by:

$$\mathrm{EiR}=1\pi\int 0\infty \mathrm{d}\omega \mathrm{Ji}(\omega)\omega. \tag{4}$$

In the FMO complex, the reorganisation energy follows a quantitative relationship with the variance of noise σ^2[28]. For site i, there is

$$\sigma \mathrm{i}2=2\mathrm{kBTEiR}, \tag{5}$$

where k_B is the Boltzmann constant. In our $\Delta\beta$ photonic model, $\sigma 2(\Delta\beta \mathrm{i})=\Delta\beta \mathrm{i}2^{-}-\Delta\beta \mathrm{i}^{-}2$. Varying the detuning amplitude $\Delta\beta_A$ from 0 to 1 mm^{-1}, we get the corresponding $\sigma^2(\Delta\beta_i)$, and meanwhile, we work out EiR by integrating the spectral density according to Eqs. (3) and (4). As shown in

Fig. 3b, the experimental data exhibits excellent linearity in accordance with Eq. (5).

Vibrational assistance

In state-of-the-art literature, the strong coupling to the the vibrational modes is believed to play an important role in energy transport[25]. The electronic coherence, vibronic coherence and vibrational coherence are found to live with different coherence time, which are 50–100fs, <500fs and >1000fs, respectively[43,44]. Our 20-mm-long photonic chip corresponds to an evolution time in the magnitude of 10ps, which goes beyond the above time scale. Still, it is interesting to investigate a vibrational assistance at the very early stage of evolution. We set an additional vibrational mode by an extra waveguide, with a vibrational coherence strength equal to the gap between the two lowest eigenstates of the 7-site Hamiltonian. As shown in Fig. 3c, the transport efficiency is clearly enhanced when coupling to the vibrational mode than without such a coupling. Note that this example uses a $\Delta\beta_A$ of 0.5 mm^{-1}. For other noise amplitudes, the enhanced efficiency via vibrational assistance always exists. See more numerical details in the Supplementary Note 7.

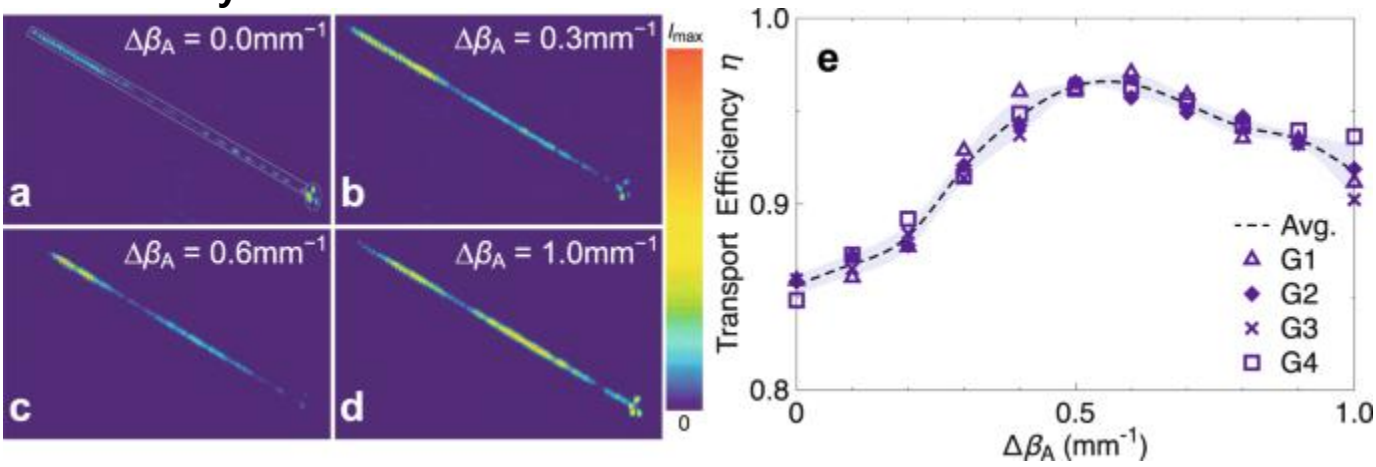
Apart from the above issues, the issues of exciton transfer[11] and energy localisation[45] are also critical when discussing transport efficiencies. We illustrate the exciton transfer process inside FMO by numerically simulating the coherent light evolution in FMO-mimic waveguide array. By analyzing the most probable excited site when varying the propagation length, we see both large detunings of the site energy that induce strong disorders, and large-scale $\Delta\beta$ noise that induce the Zeno effect, would enhance exciton localisation (see details in Supplementary Note 8). Furthermore, we show the disorder-induced enhancement of energy localisation by simulating the energy transport efficiency and the distribution of eigen-energy levels (see details in Supplementary Note 9).

Quantum simulation experiment

In experiments, we have prepared four groups of samples in the coloured noise environment, having a range of $\Delta\beta_A$ values 0, 0.1, 0.2, ..., 1.0 mm^{-1}. All samples have the noise settings that make a power spectral density consistent

with the actual FMO complex. We inject the 810 nm vertically polarised coherent light into Waveguide 6 and measure the evolution patterns using a charge coupled device. We are kind of simulating the energy packet random walk using a coherent light because when there is only one walker, the quantum coherence effects are well simulated by a coherent light field[46]. We process the figures to read out the light intensity in the seven-site part I_{FMO} and the sink part I_{sink}. Then $I_{sink}/(I_{FMO} + I_{sink})$ is worked out as the energy transport efficiency η. In Fig. 4a–d, the evolution patterns and the corresponding energy transport efficiencies for four samples in the same group of different $\Delta\beta_A$ values are presented. The experimental results for all samples are provided in Supplementary Note 10.

Fig. 4: Experimental transport patterns and the energy transport efficiency.



Three examples from the arrays formed with different $\Delta\beta_A$ values show different energy transport efficiencies. The $\Delta\beta_A$ values are 0.0 mm^{-1} for (**a**), 0.3 mm^{-1} for (**b**), 0.6 mm^{-1} for (**c**) and 1.0 mm^{-1} for (**d**). The zones for seven-site FMO complex and the sink are marked with an ellipse and a rectangle, respectively. The energy transport efficiencies are given in the bottom-left of the figures. **e** The measured transport efficiencies for samples of different $\Delta\beta_A$ values. The results for each individual sample and the averaged values are plotted in dots and a curve, respectively.

We characterise the energy transport efficiencies for all samples (Fig. 4e) and show an optimal transport efficiency for all groups up to 96% when

$\Delta\beta_A$ increases up to 0.5–0.6 mm^{-1}, followed up by an efficiency droop when $\Delta\beta_A$ further increases. Our experimental results based on the $\Delta\beta$ photonic model show that the environmental noise can assist quantum transport, just as the name ENAQT[11] suggests. We show an optimal $\Delta\beta_A$ that corresponds to an efficiency peak. This is consistent with our rich numerical analysis on the ENAQT effect (see Supplementary Note 11), where the optimal transport behaviour always occurs at an intermediate dephasing scale.

Discussion

In summary, we have fully explored the capabilities of the $\Delta\beta$ photonic model on quantum simulation of photosynthetic energy transport, with a case on the FMO complexes. By taking full advantages of the flexible arrangement of the array configuration in our three-dimensional photonic lattice, we manage a faithful layout of the coupling profile for the seven-site FMO complex. Meanwhile, the experimentally feasible $\Delta\beta$ tuning enables us to set the base site energy for different BChls in FMO, and build the non-Markovian coloured noise that simulates the power spectral density of the actual FMO complex. Through these efforts, we experimentally demonstrate an optimal ENAQT transport in the photonic lattice. We also show that the $\Delta\beta$ photonic model not only simulates ENAQT theories extensively studied during 2010s, but also can address many up-to-date interesting topics related to FMO energy transport such as the vibrational assistance.

Our quantum simulation experiment can be broadly adapted to simulating the photosynthesis processes in many other chlorophyll complexes, such as PE545, PE555, PC645, etc.[47,48,49], given their protein structures, Hamiltonian matrices and noise spectrum. Our quantum simulation experiments can give insights on the scale of noise modulation for energy transport in those chlorophyll complexes. This study may inspire applications for bioscience[50].

Our results demonstrate a powerful analog quantum simulator that can possibly be further applied to a rich diversity of researches on open quantum systems. We have noticed a recent work using digital quantum circuits to simulate the open quantum system dynamics[51]. They simulate four sites for FMO complex and require a long circuit depth, which will work on fault-

tolerant devices in the future. The quantum simulation is indeed of a broad interest[52], and strongly depends on the quantum hardware capabilities. Our integrated photonic chips have been used for various quantum simulation tasks[53,54,55] in the noisy intermediate-scale quantum era, and many can be further turned into practical modules for quantum information processing modules, such as Haar random matrix generation[36], and on-chip quantum state preserving[55], etc. This work of simulating FMO complexes essentially constructs the non-Markovian environments in photonic chips. Non-Markovian processes have been widely studied but still have many emerging applications, e.g., entanglement reactivation[56], quantum storage[57]. In order to carry out innovative exploration of non-Markovianity and its applications, strict experimental conditions are always required, and our experimental endeavor provides a way out for implementing non-Markovianity. The approach with flexible Hamiltonian mapping and controllable introduction of noise on integrated photonics is useful and worthy of further exploration on quantum simulation and quantum information processing applications.

Methods

Sample preparation

All the waveguides are fabricated using the femtosecond laser direct writing technique. We direct a 513-nm femtosecond laser (upconverted from a pump laser of 10 W, 1026 nm, 290 fs pulse duration, 1 MHz repetition rate) into a spatial light modulator (SLM) to shape the laser pulse in the temporal and spatial domain. We then focus the pulse onto a pure borosilicate substrate with a 50X objective lens (numerical aperture: 0.55). Power and SLM compensation were processed to ensure waveguide uniformity[37].

We have also characterised the quantitative control for $\Delta\beta$ detunings in the photonic lattice. We write one waveguide using a base speed V_0, and the other one using a different speed V ($V - V_0 = \Delta V$). In such a detuned directional coupler, the coupling mode method gives the effective coupling coefficient, C_{eff}, instead of C_0, the coupling coefficient for a normal directional coupler with $\Delta V = 0$. C_{eff} contains the detuning effect from $\Delta\beta$ through:

Ceff=(Δβ/2)2+C02.

Therefore, characterizing C_{eff} and C_0 gives $\Delta\beta$. See details for the characterisation in Supplementary Note 2.

Biomedicines
. 2021 Feb 22;9(2):225. doi: 10.3390/biomedicines9020225