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Review

Reversibility of irreversible aging

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ABSTRACT

Most multicellular organisms are known to age, due to accumulation of damage and other deleterious changes over time. These changes are often irreversible, as organisms, humans included, evolved fully differentiated, irreplaceable cells (e.g. neurons) and structures (e.g. skeleton). Hence, deterioration or loss of at least some cells and structures should lead to inevitable aging of these organisms. Yet, some cells may escape this fate: adult somatic cells may be converted to partially reprogrammed cells or induced pluripotent stem cells (iPSCs). By their nature, iPSCs are the cells representing the early stages of life, indicating a possibility of reversing the age of cells within the organism. Reprogramming strategies may be accomplished both in vitro and in vivo, offering opportunities for rejuvenation in the context of whole organisms. Similarly, older organs may be replaced with the younger ones prepared *ex vivo*, or grown within other organisms or even other species. How could the irreversibility of aging of some parts of the organism be reconciled with the putative reversal of aging of the other parts of the same organism? Resolution of this question holds promise for dramatically extending lifespan, which is currently not possible with traditional genetic, dietary and pharmacological approaches. Critical issues in this challenge are the nature of aging, relationship between aging of an organism and aging of its parts, relationship between cell dedifferentiation and rejuvenation, and increased risk of cancer that goes hand in hand with rejuvenation approaches.

1. Introduction

There are two principal routes to human longevity: slowing down the aging process and reversing it. Thus far, the former strategy has clearly been dominant. This is not unexpected – it is conceptually easier to come up with the ideas to slow down the emergence of age-related dysfunction (e.g. by targeting metabolism) than with the ways to rejuvenate organisms (i.e. convert them from an older to a younger state). Although researchers have largely focused on minimizing age-related deterioration, there is a fundamental need for novel strategies that can reverse the existing pathology and restore physiological function. While the ability to delay the onset of age-associated diseases (AADs) would undoubtedly be a great achievement of biomedicine, rejuvenation has the potential to alter the course of human civilization. While the clear path to the long-sought fountain of youth is as elusive as it has ever been, the opportunities the rejuvenation strategy offers, and the initial discoveries in this area, suggest that this research is worth all the effort, no matter how hard it is. However, since aging is known to be associated with the accumulation of numerous forms of damage and other deleterious changes, including those that cannot possibly be cleared up, is it even possible to rejuvenate an organism?

The issues related to rejuvenation arise from three main questions: how to define rejuvenation, how to characterize rejuvenation, and how to achieve rejuvenation. Although many longevity interventions have been developed by researchers in the field, the main questions related to rejuvenation remain unresolved. Even the definition of rejuvenation is controversial. Some define rejuvenation as a result of treating certain aging phenotypes. In this way, somehow, many interventions that have previously been defined as achieving rejuvenation may just slow down the aging process. In contrast, we can define rejuvenation as a means to move an organism from an older to a younger state. If so, only in vivo reprogramming can be treated as a known rejuvenation intervention because this is the only intervention proved mechanistically with regard to the reversal of the cells to the embryonic state. For the interventions that do not demonstrate a reversal to the embryonic state, such as rapamycin, calorie restriction, parabiosis, or senolytics, there is a need to quantify a possible rejuvenation effect, i.e. there is a need for a precise biomarker of aging.

In this review, we focus on the questions introduced above. We first examine aging and rejuvenation from the perspective of age-related deleterious changes that characterize the aging process, getting inspiration from the organisms that have variable aging phenotypes and

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those that seem to escape the aging process. We then introduce tools to assess rejuvenation, e.g. the epigenetic clock, and the biological principles behind biomarkers that assess the biological age. Finally, we dig into longevity interventions with the potential for rejuvenation, most notably heterochronic parabiosis and in vivo reprogramming, and the associated biological mechanisms.

2. Emergence of aging as a major public health threat

Until recently, diseases of aging, and aging itself, did not represent a major threat to public health. In the US, a hundred years ago the leading causes of death included pneumonia, tuberculosis, diphtheria and other currently much less harmless diseases. Advances in medicine, pharmacology and public health resulted in a dramatic decline in deaths from these infections, which in turn extended lifespan (CDC/NCHS, 2017). On the other hand, mortality due to heart disease, cancer, and metabolic dysfunction has greatly increased, especially since the discovery of antibiotics in the 1930s. Since these pathologies currently exhibit a clear age-dependent distribution within the population, they are commonly regarded as signs of aging and comprise the group of AAD.

Academic institutions and big pharma alike recognized the increasing demand for AAD treatment. An opportunity to directly act upon aging itself also attracted many scientists to this research enterprise. However, what should they specifically target? Which approach, slowing down aging or reversing it, is more feasible? Is it even possible to do it? The answers to these questions are necessarily linked to the nature of aging. The most prominent concepts of aging, such as antagonistic pleiotropy, mutation accumulation, and free radical theories, have been developed already more than half a century ago (Harman, 1956; Medawar, 1952; Williams, 1957), in turn leading to the emergence of the field of gerontology. Yet, it remains unclear what exactly aging is, what causes it and whether it can be reversed.

3. Elusive definition of aging

Most commonly, aging is regarded as an organismal phenomenon that involves an increased chance of dying and/or decreased function over time. Similar definitions are applicable to organ or tissue failure as well as to certain cell systems. However, while everybody can visually distinguish a young and an old person, and an experienced histologist may do the same for their organs and tissues, defining aging at the molecular and cellular levels has been challenging. Despite there being a compendium of classical mechanistic and evolutionary aging concepts, they seem to be too narrow to fully explain how the nano-scale life organization determines the longevity of a macro-system.

For example, Harman's free radical theory of aging proposes that partially reduced forms of molecular oxygen (reactive oxygen species or ROS) are the main drivers of aging. If so, an individual's longevity should be chiefly determined by oxygen metabolism and the associated maintenance systems. Some studies appeared to offer evidence in support of this model (Dai et al., 2014; Lagouge and Larsson, 2013). Mitochondrial antioxidants and reversed electron transport could also elicit geroprotective effects (Scialò et al., 2016; Severin et al., 2010). Despite progress in this area, the theory has failed to produce geroprotective drugs or explain the multiple phenomena occurring during aging. It also does not explain the primacy of oxidative damage as compared to other damage forms or offer a satisfactory evolutionary explanation (Gladyshev, 2014; Hekimi et al., 2011).

Incompleteness of classical mechanistic theories may be attributed to there being no single cause, or even no single main cause, of aging (Fig. 1). Aging is a complex process involving various mechanisms that lead to the accumulation of subcellular, cellular and intercellular damage as well as other age-related deleterious changes, together representing the organism's deleteriome (Gladyshev, 2016). As an organism progresses through its life, its deleteriome increases. The rise in the organismal deleteriome is what appears to determine the biological age of this organism. The deleteriome concept represents an alternative to the phenotype-based thinking of aging. While it may be unclear what an old molecule or cell phenotype is, the damage may be easier to define. This approach necessitates the use of complex molecular criteria (e.g. comprehensive transcriptomic, proteomic and metabolite profiles, or even better their integration), as opposed to the use of simple aging markers (e.g. membrane peroxidation, telomere length, etc.).

4. Irreversible aging

There are organisms that age and there are those that do not. The former category includes the majority of multicellular and many unicellular organisms, and the latter includes symmetrically dividing unicellular organisms and some multicellular organisms that can replace all their cells from stem cells. Most of such multicellular organisms (such as sponges and jellyfish) are primitive in their organization and in this way are related to seemingly immortal unicellular organisms. This suggests that the first multicellular organisms may have been ageless, and they lost this property while becoming more complex (Petralia et al., 2014). Developing highly specialized and irreplaceable systems renders complex animals more susceptible to critical failures.

Humans are one such example. We evolved neurons that fully differentiate already during embryonic development, cardiomyocytes that are largely not replaced during the adult life, elements of the eye (e.g. lens) that can only add new material over time and are unable to remove old components, teeth that cannot be replaced in the adult stage, and skeleton that is also irreplaceable once fully grown. Damage accumulating in these and other structures as well as the loss of fully differentiated, irreplaceable cells such as neurons and cardiomyocytes should be viewed as permanent. As such cells and structures accumulate damage and dysfunction, they will age, and if irreplaceable elements of an organism age and die, then the organism as a whole will age as well. Therefore, human aging may be considered as irreversible, even if some cells in the organism can be replaced and some damage removed. This can be illustrated by the metaphor of a car. If its engine cannot be replaced, a car that is driven will necessarily age and eventually fail, even if other parts of the car, e.g. tires, can be replaced.

How can the apparent irreversibility of human aging be reconciled with the emergence of research on rejuvenation? What does it mean to rejuvenate an organism if some of its elements age irreversibly? There are currently no clear answers to these questions. To begin addressing them, we will need to understand what rejuvenation is at the molecular and cellular levels.

5. Epigenetic markers of aging

One of the contributions to aging comes from epigenetics. With age, the epigenetic landscape of cells changes dramatically, reflecting the patterns of damage accumulation. These changes are also reflected in chromatin remodeling and histone modifications. The large number of such changes and the ability to track them provide important insights into the aging process.

For example, extensive analysis of human DNA methylation (DNAm) resulted in the "epigenetic clock", a set of CpG sites that represent a mathematical model that can serve as a biomarker of human aging. Interestingly, such clocks can be made regardless of the tissue of origin, e.g. the first human multitissue epigenetic clock is defined by the weighted average of 353 CpG sites (Horvath, 2013). Other clocks may represent the aging of a particular tissue, e.g. the first blood clock is defined by 71 CpG sites (Hannum et al., 2013). In these and other clocks, changes in methylation levels strongly correlate with the chronological age of a person. The resulting models may predict a person's age with a mean error of only 3–5 years. Mechanistically, it remains unclear if changes in methylation at these specific sites is the consequence of the active epigenetic machinery or the result of

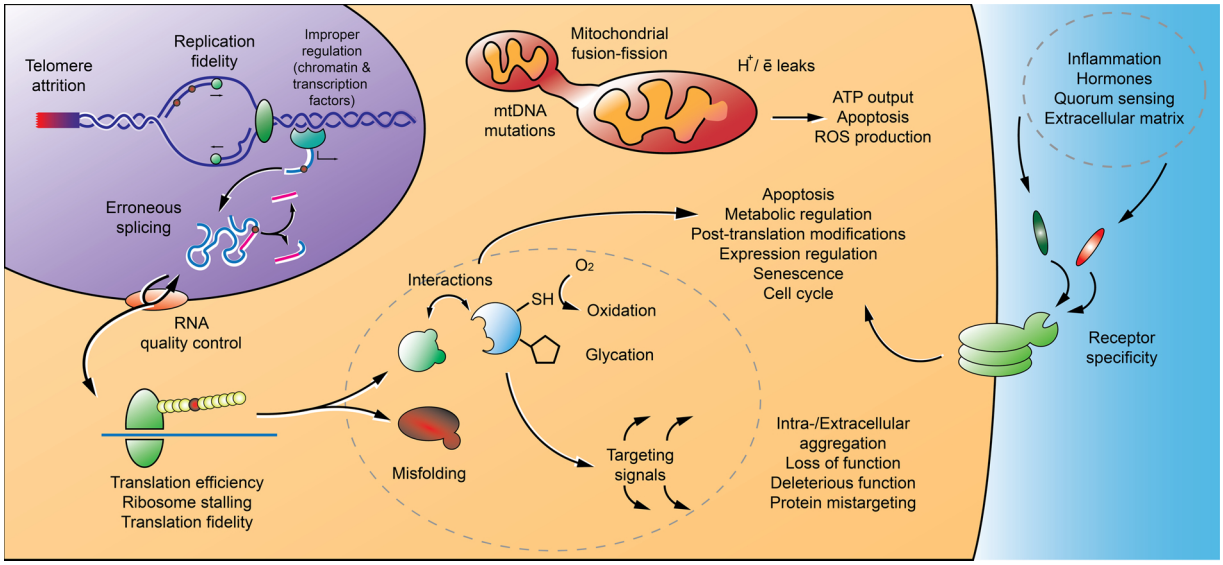

Fig. 1. Cellular and organismal aging is linked with numerous molecular processes. These processes include information transfer (e.g. DNA replication, transcription, translation, signal transduction), physical manifestation of information (e.g. splicing, RNA quality control, folding, post-translational modifications, protein targeting, receptor triggering, telomere attrition), stochastic processes (e.g. macromolecule oxidation, protein glycation, protein interactions, membrane transport) and other aspects of aging.

unrepaired DNA damage, and whether DNA methylation changes are causal to aging or are true biomarkers with no influence on the aging process. At this point, the clocks are more of a convenient molecular tool rather than an applied theory (Horvath and Raj, 2018).

Similar to the human clocks, a robust predictor of mouse biological age was recently developed that is based on 90 CpG sites derived from blood DNA methylation profiles. The resulting clock could be used to evaluate the age of mouse cohorts and detect the longevity effects of calorie restriction and several gene knockouts (Petkovich et al., 2017). Several clocks were also developed based on other mouse tissues, including multi-tissue and liver clocks (Stubbs et al., 2017; Wang et al., 2017). An important role of epigenetic marks in aging is also supported by experimental evidence. For instance, a mouse liver clock could detect the effects of geroprotective interventions such as caloric restriction and rapamycin treatment as well as predict slower aging of long-lived mouse lines (Wang et al., 2017). Other clocks in mice also identify aging-related effects of diet and hormones (shorter lifespan and greater predicted age of mice subjected to high-fat diet and female mice subjected to ovariectomy) (Stubbs et al., 2017).

DNAm clocks continue to be improved and diversified. For example, a recently developed PhenoAge clock assesses *phenotypical* age rather than *chronological* age (Levine et al., 2018). Phenotypical age is a metrics derived from a set of medical biomarkers and is indicative of AAD risks and all-cause mortality rate. In a sense, defining age as a function of deleterious processes may be more practical than defining it as a function of time, as it carries more biological meaning. Interestingly, only 41 out of 513 CpGs included in PhenoAge are also present in the original Horvath's clock, which hints at there being a significant difference between the age as a measure of time and the age as a measure of withering.

6. Epigenetic machinery involved in aging

If epigenetic patterns may reflect the aging process, can they be uncoupled from other molecular changes in the cell? Does epigenetics represent the root of aging? There is a strong reason to believe that strictly epigenetic mechanisms may regulate lifespan. Arguably, the most colorful illustration of this notion is the honey bee (*Apis mellifera*) caste differentiation mechanism. Queen bees can live up to 8 years (normally 3–5 years), while worker bees usually last only 6 weeks during foraging periods and may reach 0.9 years during cold season (Haddad et al., 2007). Despite this dramatic difference in longevity, queens and workers share the same genome. However, their epigenomes are different. More specifically, queen-worker speciation involves a DNA methylation mechanism, as treating larvae with the siRNA targeting the methyltransferase Dnmt3 leads to the queen phenotype (Kucharski et al., 2008). Interestingly, this artificial manipulation turned out to mimic a naturally occurring process as the queen-to-be larva are fed large amounts of the royal jelly, which both reduces Dnmt3 expression and activity (Shi et al., 2011). Thus, DNA methylation may lie at the base of the bee caste system and queen longevity. These insects can be said to have mastered the art of applied epigenetics that biologists are only starting to employ.

DNA methylation is not the only epigenetic footprint aging leaves on the genomes. Chromatin remodeling and histone modification also change dramatically with age. These changes may start at the level of biosynthesis, as suggested by the study that found H3 and H4 synthesis to be 50% lower in fibroblasts from a 92-year-old individual compared to cells from a 9-year-old child (O'Sullivan et al., 2010). It was further hypothesized that telomeric shortening produces DNA damage response, which destabilizes cell's capacity to properly control chromatin remodeling by reducing histone and chaperone synthesis. Although a link between telomeric and epigenetic theories would be of interest, it requires further experimental analyses (Galati et al., 2013; Song and Johnson, 2018). Senescent cells are also characterized by chromatin decondensation at centromeric regions, as well as chromatin changes at telomeric regions (Ren et al., 2017). Interestingly, the method used to visualize these chromatin changes has yielded a novel aging marker in humans – ribosomal DNA repeat attrition, which was previously reported only in yeast (Ganley and Kobayashi, 2014).

Attempts to understand the role of epigenetics in aging strike two major issues. (i) The cause-effect relationship between epigenetics and aging is not well established. It remains unclear whether epigenetic marks represent markers or drivers of aging (or both). (ii) Even if an epigenetic modification directly affects longevity, it is often unclear, if the effect can be attributed to the epigenetic machinery *per se* or to the corresponding changes in gene expression.

7. Progeroid organs

Mammalian tissues and organs may unequally contribute to aging and may also age with different rates. The concept of particular organs contributing more to the aging process implies that lifespan is an evolutionary derived program that can be hacked by targeting these organs. The search for such targets began decades before biogerontology became mainstream science. At the beginning of the 20th century, when the molecular basis of life was still largely unknown, some people already claimed to have figured out why people age and how to stop it. After Brown-Sequard reported improved vigor following testis extract injections, this organ was considered a key to aging (Stambler, 2014). As a result, rejuvenation surgeries targeting the reproductive system gained popularity in the 1910–1920 s. Sterilizing operations and grafts of chimpanzee testes were particularly popular among the old and wealthy. Such famous doctors as Steinach, Lichtenstern and Voronoff made a fortune on these harmful operations. Experiments with synthetic testosterone in the 1930s led to the denouncement of rejuvenation surgeries on the reproductive system (Kozminski and Bloom, 2012). More broadly, the physiological approach to rejuvenation studies was compromised. However, the concept of progeroid hormones and organs still persisted.

The aging organ hypothesis reemerged in the 1970s when various experiments showed the hypothalamic hormones to affect organismal phenotypes related to life histories (Everitt, 1973). Even now some scientists consider aging to be executed via the nervous system with the hypothalamus being a key regulator. This idea is based, for instance, on the research showing that inhibiting the NF-kB pro-inflammatory pathway in murine mediobasal hypothalamus leads to up to 20% lifespan extension and amelioration of aging phenotypes. Gonadoliberin also produces a similar effect, which seems to prove an important role of hypothalamus in regulating lifespan (Zhang et al., 2013). Hypothalamus is undeniably a master regulator for many physiological processes, and adjusting its hormone-releasing activity may delay the onset of some AADs. However, many processes that elevate the deleteriome come from other organs and have additional extrinsic sources (UV-caused mutations, dietary effects, stress responses). In such circumstances, aging cannot possibly be completely controlled by a major organ or a pathway. Evolutionary logic also supports this idea. If an organ drives the aging process, selection may be relaxed on other organs and systems until they more or less synchronize in their impact on aging with that organ. Therefore, while the search for organs and tissues that drive aging may provide new therapeutic targets for AADs or even help extend lifespan, it has a limited use for radical changes in lifespan.

8. Heterochronic parabiosis

Heterochronic parabiosis (HP) experiments in rats in 1972 opened an entirely new avenue in the search for the molecular basis of aging (Conboy et al., 2013). HP is a surgical procedure of grafting syngenic animals of different age together, whereby they share circulatory systems, allowing the old animal prolonged access to the blood of the young. The original study showed that older parabionts tended to live longer than paired animals of the same age (Ludwig and Elashoff, 1972). Since then, many scientists have asked what could possibly induce HP beneficial effects. Could there be any juvenile protective factors in the young animals' blood? And if they exist, do they support rejuvenation or just slow down the aging process?

After extensive HP research, one youthfulness factor has been described. This proposed effector molecule is GDF11. Its levels were reported to decrease with age (Poggioli et al., 2016), and older animals injected with recombinant GDF11 could restore olfactory (Katsimpardi et al., 2014) and motor (Sinha et al., 2014) functions. However, other research groups were unable to reproduce these effects (Egerman et al., 2015; Smith et al., 2015b). There are also studies that show GDF11 suppresses neural stem cell proliferation and induces muscle degeneration (Egerman et al., 2015; Hinken et al., 2016; Williams et al., 2013; Zimmers et al., 2017). Another molecule proposed to mediate HP rejuvenation is oxytocin (OT). OT is a peptide neurotransmitter, produced mostly by the hypothalamus, that regulates social behavior and maternal bonding. Apart from its behavioral functions, OT is a hormone that affects energy consumption in multiple tissues (Chaves et al., 2013). Older mice show decreased levels of circulating OT compared to younger mice. This decrease apparently affects muscle tissue the most as the number of OT-receptors in muscle satellite cells is also diminished with age. Subcutaneous OT injections could rescue impaired muscle regeneration in sarcopenic mice, further supporting OT's role as a potent HP effector molecule (Elabd et al., 2014). Further research, however, is needed to prove the role of OT, other individual factors in the circulation, or their combination, in the HP effects.

These challenges notwithstanding, HP remains a promising experimental model. Blood milieu changes with age so greatly, that its biochemistry can be used to produce age predictin models of comparable accuracy to DNAm clocks (Putin et al., 2016). Various studies reported that stem cell and progenitor cell activity is highly dependent on the systemic environment. Though it is unclear what factors form this environment, regenerative capacity has been shown to be restored via Notch pathway activation and chromatin remodeling (Conboy et al., 2005). HP is particularly interesting as the observed rejuvenation does not require genetic intervention or artificial epigenetic reprogramming, and once the effector molecules are found and verified they can be synthesized and may become the actual medicine. Despite there being limited information on HP effectors, the pathways affected by HP have been reported (Brack et al., 2007; Conboy et al., 2005), and stimulating them might be a way toward rejuvenation. A screening for compounds affecting the deleteriomes of younger organisms is another strategy to identify new targets for pharmacological rejuvenation. However, it is unlikely that a specific molecular pathway stands behind the HP phenomenon. The identified juvenile protective molecules might represent differences in the systemic milieu, by-products of metabolism or signatures of aging rather than the cause(s) of aging or youthfulness.

It is important to emphasize that HP rejuvenation mechanisms are generally obscure. While it cannot be excluded that the effect due to a single "silver bullet" molecule, the youthfulness factor is unlikely to be singular. The old blood may also bear pro-aging factors that are diluted when mixed into younger animals' circulation. For example, aged human blood has been shown to contain senescent immune cells compromising immunity (Chou and Effros, 2013). Filtering out these cells from the bloodstream has proven to be a potent method to target immunosenescence (Rebo et al., 2010). In fact, old blood transfusion has been shown to lower muscle, hippocampal and liver performance and proliferative properties in younger mice. Moreover, young blood transfusions do not necessarily raise the same properties (e.g. muscle performance without trauma) in older mice (Rebo et al., 2016).

The negative effects the old blood exerts on younger animals have been linked with age-associated activation of innate immunity— the so-called inflammaging. ß2-microglobulin (B2M, MHCI subunit and inflammation marker) seems to be one of the mediators of HP negative effects on younger animals. It is elevated in older plasma and becomes elevated in multiple tissues of younger animals upon older blood exchange (Rebo et al., 2016). B2M has also been shown to directly cause hippocampus-dependent cognitive function decline in mice upon systemic or hippocampal injection (Smith et al., 2015a). Other inflammation regulating molecules that are elevated in older blood (TGFß1, AngII, IL6 etc.) might produce similar effects (Xia et al., 2016). The overall mixture of these factors creates proinflammatory milieu both locally and systemically. Although key molecules that "age the young" during HP are not yet identified, the involvement of innate immunity in this process is now well established. Therefore, HP rejuvenation can be at least partly attributed to damage dilution that cannot be reproduced by drug administration. If dilution is a major

contributor to this phenomenon, then HP studies might never deliver satisfying results.

9. Reprogramming may reset the age of some cells in an organism

An important aspect of aging is the misregulation of gene expression which arises from the shift in the epigenetic state of the cell. For example, epigenetic marks on the DNA can be added or removed by intrinsic mechanisms and can be affected by extrinsic factors (e.g. smoking, diet, hypoxia) or developmental programs (differentiation). Once altered, such epigenetic changes can remain following many cell divisions, and if these marks are altered in the germline, these changes may be inherited by the offspring. Epigenetic inheritance is opposed by erasing the marks during germ cell maturation (Hajkova, 2011) and fertilization (Morgan et al., 2005). Epigenetic mark clearance following conception returns genome to its ground state, ensuring zygote totipotency. It should be noted, however, that some marks can evade germline reprogramming and result in epigenetic inheritance, transgenerational in certain cases (Heard and Martienssen, 2014).

Thus, from the epigenetic point of view, fertilization is the moment when an organism's age is reset to zero. Somatic cells do not follow this strategy. However, thanks to the Yamanaka's Nobel-winning studies, epigenetic reprogramming has become a routine strategy that can be used to reset the age of differentiated somatic cells. Many cell types could be returned to its ground state with a cocktail of four transcription factors: Oct4, Sox2, Klf4, and c-Myc, (Yamanaka factors, hereafter called OSKM) (Takahashi and Yamanaka, 2006). Epigenetic reprogramming can be used to obtain induced pluripotent stem cells (iPSCs) from diverse cell types for a wide use in biomedical research, e.g. for treating injuries and diseases (Singh et al., 2015). Its progression to clinic seems to be only a few years away with numerous therapies currently going through trials (Trounson and DeWitt, 2016). Most of these therapies rely on isolating patient's differentiated cells (typically fibroblasts), reprogramming them to iPSCs, and then differentiating them to a cell type needed for transplantation. An alternative method, called direct reprogramming, is sometimes used to skip the iPSC stage and directly convert one cell type to another. These techniques may let doctors obtain transplants with perfect histocompatibility. In each case, reprogramming should be thoroughly optimized and tested but the opportunities for plausible cell type conversion are rapidly expanding (Wyatt and Dubois, 2017).

A practical issue frequently discussed in the epigenetic reprogramming literature is whether re-differentiated cells remember their origin. Rephrased in the context of gerontology, this question asks whether reprogramming erases all aging hallmarks (epigenetic marks, telomere length, oxidative damage, etc.) to actually rejuvenate cells. At first, cellular senescence was considered to be a major obstacle to iPSC reprogramming as the procedure led to upregulation of factors involved in permanent cell cycle arrest. Moreover, direct reprogramming has been found to preserve aging phenotypes via up-regulation of p53, p16 and p21 involved in cellular senescence (Banito et al., 2009; Mertens et al., 2015). Thus, epigenetic reprogramming for therapeutic purposes was impossible for subjects in their late life. However, protocol optimization rendered cell cycle arrest reversible and iPSCs derived from centenarian tissues have been proven to be indistinguishable from human embryonic stem cells (Lapasset et al., 2011).

The rejuvenation effect in iPSCs is consistent with the analyses by DNA methylome clocks, which display zero (or even negative) age for reprogrammed cells (Horvath, 2013; Petkovich et al., 2017). An important consideration is that several studies reported that iPSCs can be distinguished from embryonic stem cells. For example, careful examination of the iPSC methylome has shown that they retain residual memory of the donor's age (Lo Sardo et al., 2017). Certain CpG sites preserve their age-specific methylation status even after 100 passages following reprogramming, which allowed distinguishing iPSCs produced from young and old donors. iPSCs also possess the memory of their initial tissue. For example, iPSCs have different teratogenic, hematopoietic and osteogenic potential based on their tissue of origin (Kim et al., 2010; Miura et al., 2009). All these findings suggest that epigenetic reprogramming does not fully return differentiated cells to their ground state, but rather approximates it. What predefines the level of accuracy and how exactly epigenetic rejuvenation is orchestrated remains unknown. This information is critically needed in order to assess the molecular basis of rejuvenation. An important question is also how rejuvenation and dedifferentiation relate to each other during cell reprogramming. Can the two programs be separated, i.e. can one obtain a rejuvenated cell that has not lost its lineage characteristics completely. A distinct combination of Yamanaka factors and the use of other factors, both genetic and chemical, may be used to address these questions.

Although the Yamanaka's approach to cell dedifferentiation currently dominates the field, it is not the only way to produce pluripotent cells. Surprisingly, strictly geometrical constraints can lead to stemness in fully differentiated cell cultures. Lateral confinement in 1:5 rectangular micropatterns has been shown to promote the transition of single layer fibroblast cultures into dedifferentiated spheroids with 20% success rate (Roy et al., 2018). Interestingly, gene expression analyses of the resulting spheroids indicate that Sox2 and Oct4 (as well as a number of other pluripotency-associated genes) are upregulated during the transition. Epigenetic age of spheroids has not been assessed yet, so it is unclear whether the actual rejuvenation takes place. Although they have been shown to differentiate into neuron-like structures, more extensive research on spheroids' differentiation potential is required. This unique example of mechanical reprogramming proves the immense role of cellular contacts in cell identity and offers an unexpected alternative to epigenetic reprogramming.

Another way to set a genome age to zero is somatic cell nuclear transfer (SCNT), a method to reprogram the nucleus of a differentiated somatic cell with the use of oocyte cytoplasm (Matoba and Zhang, 2018). The resulting chimeric cell can form an embryo or produce embryonic stem cells with the age apparently erased. However, the issues of whether all aging epigenetic marks are completely removed during the SCNT procedure, and whether the aging process of the resulting cloned animals is normal, remain controversial (Burgstaller and Brem, 2017; Loi et al., 2016).

10. Practical roadblocks to epigenetic rejuvenation

Despite its overwhelming effectivity in vitro, reprogramming has severe limitations in vivo due to a high risk of serious side effects, e.g. massive cell dedifferentiation may lead to the loss of organ function and to teratoma formation (Abad et al., 2013; Ohnishi et al., 2014). Pluripotent cells may also acquire mutations in p53, other tumor suppressor genes, and oncogenes that promote cell proliferation. It is thus critical to carefully genotype such cells prior to their use in the clinic (Merkle et al., 2017). Moreover, the reprogramming proteins themselves possess oncogenic properties which produce a risk of somatic tumor formation (Riggs et al., 2013). Researchers are currently trying to overcome these obstacles and develop medical epigenetic reprogramming strategies (Fig. 2).

One way to apply reprogramming for organismal rejuvenation is the utilization of short-term cyclic expression of Yamanaka factors in mice (Ocampo et al., 2016). More specifically, induced expression of these four transcription factors throughout the body was reported to lead to an improved regenerative capacity of the pancreas and muscle following toxic injury, and even to extended (1.3-fold increase) median lifespan of progeroid LAKI mice. Interestingly, such cyclic reprogramming has been demonstrated in vitro to decrease fibroblasts' epigenetic age before cells reach the pluripotent state (Olova et al., 2018), although another report found no rejuvenation in cells with aborted OSKM expression (Göbel et al., 2018). The finding with LAKI mice does not yet represent true rejuvenation of mice, as it involved a reversal of

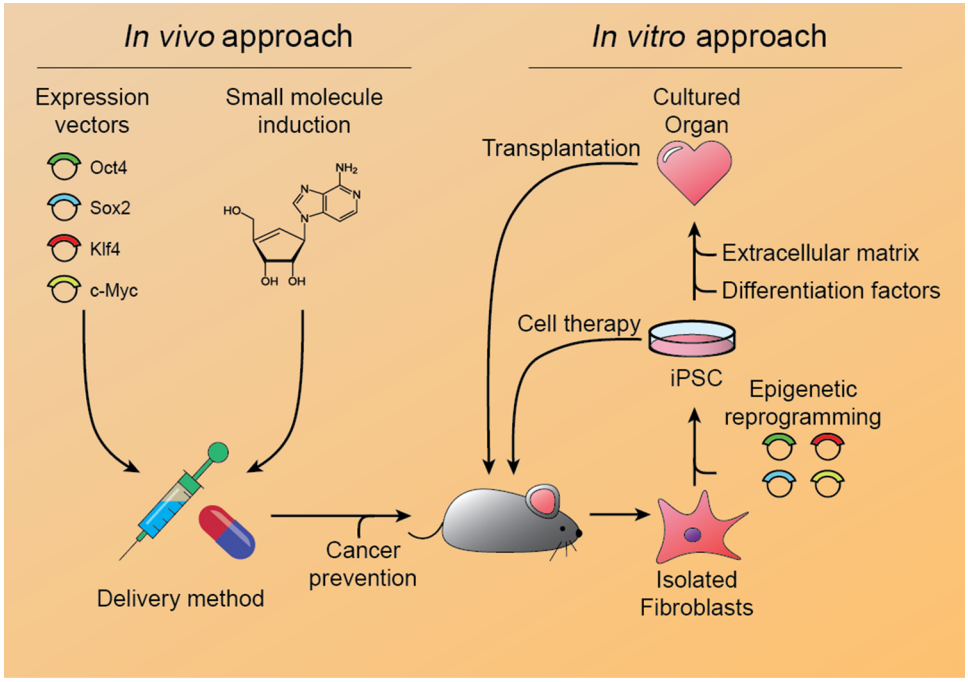

Fig. 2. Approaches to iPSC therapies. in vivo methods carry a risk of cancer development, but leverage an organism's self-organization potential. On the other hand, in vitro methods are cancer-free, but rely on technologies that allow the formation and growth of artificial organs. Cell therapies lie in between these alternatives as differentiation happens *ex vivo*, while tissue formation occurs in vivo.

progeroid phenotypes. Nevertheless, the study demonstrated that the administration of Yamanaka factors may be a viable strategy and has created a platform for future studies. As an example of this strategy, local OSKM expression in mouse cutaneous wounds was reported to result in reduced scar tissue formation (Doeser et al., 2018). Thus, partial cell reprogramming in situ may improve tissue healing without the risk of tumor formation.

Another possibility to bypass the teratogenic properties of induced Yamanaka factor expression in humans is the use of small molecules. OSKM expression may be induced not only directly via genetic vectors, but also chemically. A cocktail of small molecules has been identified that is able to substitute the OSKM expression vectors in mouse fibroblasts to derive iPSCs (Hou et al., 2013). Although similar cocktails have recently been reported to achieve *in-vivo* cardiac reprogramming, it should be noted that this method has not been applied directly for rejuvenation in vivo. Therefore, side effects, including tumorigenesis, may render chemical induction inappropriate for in vivo systems in the absence of proper targeting. Other small molecules having the potential of eliminating teratomas, such as metformin, have also been used in iPSC transplant model. However, this compound can only limit tumor growth, and the effect achieved is far from being tumor-free rejuvenation (Vazquez-Martin et al., 2012). So far, the only teratoma-free reprogramming therapy has been achieved in the genetically modified mice discussed above (Ocampo et al., 2016). Developing strategies to produce iPSCs in vivo is a challenging task, as reprogramming involves oncogene activation. Nonetheless, there are techniques that may help obtain iPSCs in the environments supported by cancer resistance (Garcia-Cao et al., 2012). The existence of such techniques promises to provide a test platform for more sophisticated therapy settings.

Although animals genetically modified to support cancer resistance are a viable solution for use in model organisms, genome manipulation is a risky strategy for human reprogramming therapies. Due to their borderline legal and ethical status, a clinically acceptable Yamanaka factor therapy should involve non-integrative agents. Since the original Yamanaka discovery in 2006, a compendium of delivery methods has been developed including non-integrative viruses (Fusaki, 2013), membrane-permeating proteins (Zhou et al., 2009), “minicircle” vectors (plasmids free of bacterial DNA) (Jia et al., 2010), mRNA transfection (Warren et al., 2010) and others. Recently, adeno-associated virus (AAV) has been applied to deliver Yamanaka factors in mice. However, the effect of partial reprogramming has not been tested in this model (Senís et al., 2018). Mimicking short-term cyclic induction of Yamanaka factors in vivo with these delivery systems is undoubtedly a challenging task.

Despite all forthcoming efforts, in vivo reprogramming might be more favorable than the use of iPSC-derived cell culture to produce transplants. iPSC-based transplantology requires *ex vivo* cell differentiation, organization into functional units, surgery and postsurgical adaptation, while in vivo application of Yamanaka factors takes advantage of existing organ structures and cell niches that direct iPSC self-organization. However, OSKM administration bears high tumorigenic risk, which must be reduced for any viable clinical application. Gene therapy may provide the platform for this, but as of today human reprogramming remains only a hypothetical geroprotective treatment.

It should be noted that the underlying mechanisms of iPSC-driven rejuvenation may not be limited to iPSC-derived progenitors and differentiated cells, but also involve the paracrine effects of the iPSCs on neighboring cells. Various RNA species, proteins, and even entire organelles, passed via the secretory pathway, exosomes, cytonemes or other types of cell-to-cell contacts, may improve the procedures and help rejuvenate donor cells and tissues. Thus, cell-free approaches based on iPSC-derived purified components should be also kept in mind as a potentially less harmful substitute for iPSC induction or transplantation.

11. Telomerase activation

Telomeres are the DNA regions at the linear chromosome termini, having specific strand and chromatin structures. Inability to replicate telomeres leads to their shortening after each cell division, which is thought to define the Hayflick limit of cell division: upon losing a critical portion of telomeres, a cell is unable to divide. Telomerases are the enzymes that generate telomeric repeats using RNA templates and can allow certain cells to surpass the Hayflick limit. However, in multicellular organisms, telomerase expression is generally restricted to prevent malignant transformation. Telomere shortening was proposed to be a key factor in aging and tumorigenesis in the 1970s by Alexey Olovnikov, and these ideas received experimental support in the 1990s

(Feng et al., 1995; Olovnikov, 1973). It is often discussed that telomere shortening may limit tissue turnover and contribute to aging, whereas telomerase activation may contribute to rejuvenation, provided it does not lead to tumorigenesis. However, many cells in an organism do not divide and do not shorten telomeres, so telomerase is not relevant to the aging of these cells. Thus, telomerase cannot explain aging or rejuvenation of organisms.

It was also established that larger animals such as primates tend to choose an onco-protective strategy of repressing telomerase, whereas smaller animals have a lower risk of cancer due to having fewer cells in their bodies and can afford the benefits of active telomerase. Interestingly, there is apparently no correlation between telomerase activity and lifespan (Gorbunova and Seluanov, 2009; Seluanov et al., 2007). The telomerase aging model is currently shadowed by other models, as reprogramming has the potential to restore telomere length and reactivate telomerase. However, the telomeric approach to rejuvenation remains an active area of research. A recent study has shown an improvement of healthspan in C57BL/6 mice upon viral telomerase expression (Bernardes de Jesus et al., 2012). Constitutive expression of telomerase reverse transcriptase (TERT) in cancer-resistant mice has also led to the lower incidence of inflammatory diseases (associated both with skin and gastrointestinal tract), glucose intolerance and display greater neuromuscular coordination (Tomás-Loba et al., 2008). Obtaining the same results in humans would be challenging, and the actual rejuvenation would be even more difficult, as telomerase clears only one form of DNA damage while leaving all other deleteriome components untouched. Nevertheless, in 2015, Bioviva's CEO Elizabeth Parrish surprised the world by applying her company's experimental telomere lengthening therapy to herself (Dara Mohammadi, 2016). This endeavor has been perceived by many as a marketing campaign and by others as dangerous and unthoughtful.

12. Wholistic aging

Reductionist approaches offered multiple helpful insights into the aging process, especially at the molecular and cellular levels. However, despite its broad utility, reductionism has some limitations (Lakatos, 1976), and this is particularly relevant in the case of gerontology, as aging is a strictly organismal phenomenon that should not be viewed as a scaled up cellular deterioration. Moreover, even though biological wholism can be regarded as an unnecessary and even counterproductive overcomplication, it can be used to derive functional insights into the aging process.

One such concept is the existence of a general regenerative potential within an organism. While the cellular regenerative potential is extensively studied and even can be manipulated, modern biology has been struggling to properly realize it in complex systems. Modern advances in *ex vivo* organ cultivation and transplantology are just a reductionist shortcut to organismal youth, while the wholistic approach suggests that the most efficient way to rejuvenate an organism is to mobilize its intrinsic regenerative potential. Sometimes the wholistic approach to gerontology means multivariate analysis leveraged by clustering and classification techniques (Gerstorf et al., 2006). In this review, however, the wholistic approach is viewed in the experimental context, which primarily includes studies on self-organization and regeneration.

A recent study on planarians has found that proper regeneration requires organism-level orchestration (Atabay et al., 2018). While planarian organs themselves produce proteins that direct cell progenitors, their whole organism also produces protein gradients that inform all cells on their position in the body. Distorting the latter mechanism has been elegantly displayed to produce animals with multiple ectopic eyes and no memory of their initial normal anatomy. This study indicates that very little is known about dynamic self-organization of biological systems. It also suggests that it would be naïve to believe that key insights into regeneration and rejuvenation can be obtained while ignoring aging of the whole organism.

In support of this argument, several studies point towards a similar positioning mechanism involved in mammalian regeneration. More specifically, fibroblasts have been shown to have distinct expression profiles depending on their position in the human body (Rinn et al., 2006). The identified patterns include 337 genes and allow locating a fibroblast's origin site among 43 sampled sites. These ubiquitous cells preserve positional information extensively used during embryo development. Considering fibroblasts' crucial role in matrix formation and wound healing, this information is likely to be needed for proper mammalian regeneration.

Positional information does take part in mammalian regeneration as shown in the murine digit tip model. BMP2 induces regeneration of digit tips amputated at P2 or P3 levels (second or terminal phalangeal element, respectively). However, cells differentiate distal to proximal in P3 and proximal to distal in P2 (Yu et al., 2012). The same morphogenic factor producing two different responses is a clear indication of positional information regulating digit restoration. Thus, deciphering organismal coordinates is likely to be required to gain control over mammalian regeneration. However, positional information is definitely not the only cornerstone. Blastema (dedifferentiated cellular mass in wounded sites) formation in fish and amphibians such as axolotls is a major step in post-injury regeneration, but is not yet clearly understood. More specifically, it remains a mystery whether it is possible to induce blastema formation instead of scarring in mature mammals. Unlike human adults, human fetus can genuinely regenerate bone and skin injuries during the second trimester (Yates et al., 2012). This effect is attributed to the lack of inflammation and fibroblast migratory and secretory properties. These findings indicate that humans have not lost regenerative potential completely, and that it may be induced, akin to the conversion of adult cells to pluripotency upon reprogramming with Yamanaka factors. Such an induction may take organ- and purpose-specific approaches. For example, transducing cutaneous ulcers with DNP63 A, GRHL2, TFAP2A, and MYC has been shown to mobilize mesenchymal cells and differentiate them into keratinocytes, which rescued wound closing. Unlike the Yamanaka cocktail, these transcription factors have not displayed teratogenic properties while maintaining high proliferative activity (Ankawa and Fuchs, 2018; Kurita et al., 2018).

It appears that blastema-type regeneration is a multistage process composed of systematic crosstalk between various cell signaling pathways, and manipulating it suggests introducing sequential interventions at all the steps (probably including wound positioning) (Simkin et al., 2015). Determining how the organism as a whole is involved in even minor acts of regeneration is indeed a challenging aspiration. But the data suggest it may be necessary to fully uncover the intrinsic regenerative potential in humans.

13. Outlook

It is clear that to understand the potential for reversing the aging process, even in some parts of the organism, we need to understand the nature of aging and identify the targets for intervention. Over the years, numerous mechanistic theories of aging have been advanced. Most of them have some merit but seem to be incomplete. Although experiments have led to lifespan extension and, in some cases, to improvements in healthspan in various model organisms, whether true rejuvenation has ever been achieved through these approaches remains unclear. Many damage-centric theories (telomeric, free radical, DNA damage, etc.) focus on single aspects of aging, but aging is a systemic, organismal process. It has numerous molecular causes and may manifest itself in very different ways. The cumulative deleterious processes compose an organism's deleteriome, and many of its components cannot be cleared from fully differentiated, non-renewable cells.

The deleteriome concept may be illustrated with the metaphor of car aging. Driving a car leads to wearing out its brakes and tires, engine

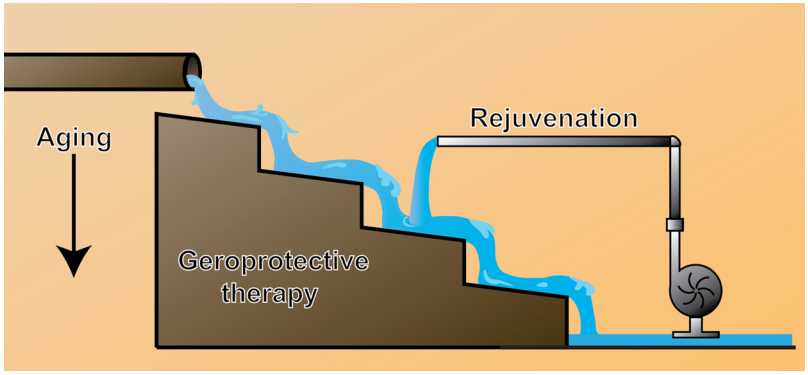

Fig. 3. Aging is akin to gravity: it is a fundamental force of nature that can never be truly reversed. Just as gravity directs rivers, aging inevitably leads to impaired biological function. Geroprotective therapies can slow it down, just like dams and canals slow down rivers, but never reverse it. Rejuvenation technologies, on the other hand, decrease age and are more appropriately compared to water pumps. Nonetheless, even rejuvenation technologies do not reverse aging, just as water pumps do not negate gravity.

problems, body rusting, windshield cracking and many other types of cumulative damage. Looking into the causes of this damage we notice friction, oxidation, rigid body deformation, overheating, etc. Thus, a car's deleteriome unites the processes containing fundamental causes and their visible outcomes. Telomere lengthening and many other approaches seem to be relatively minor issues compared to the amassment of components of the deleteriome — just as minor as applying rust remover is to a car's deleteriome. Moreover, such minor changes only affect the outcome of a deleterious process, leaving its cause intact. In fact, the causes are so fundamental it is practically impossible to target them all. The biological deleteriome also contains very basic causes: imperfect information transfer, irreversible non-catalytic reactions, imperfect enzyme specificity, unbalanced energy production and consumption. These are essential attributes of any living system and altering them all together is practically implausible at the current state of science.

The way to achieve rejuvenation in biological systems is analogous to the strategy of parts replacement employed by car mechanics. Elaborating on the car metaphor, different parts have the varying rate of deterioration and wear and tear and it is sensible to first replace those most unreliable. Reprogramming is a gateway technology that may allow people to obtain individual "spare parts". iPSCs produced with Yamanaka factors are akin to donor's embryonic stem cells and can be used to replace senescent cells. However, it is still unclear which is more efficient: isolating iPSC and organizing them in organs *ex vivo* or in vivo administration of OSKM, as both these paths are still under development. The former option requires a full control over iPSC differentiation into transplant-quality organ or tissue, and the latter — discovering a way to deliver and activate Yamanaka factors in patient's cells avoiding tumorigenesis.

A third option also exists that aims to combine the advantages of both while dodging their drawbacks. A procedure called interspecies blastocyst complementation allows obtaining viable pig-human chimeras via injecting donor's iPSCs into pig's blastocyst (Wu et al., 2017). Although the final quantity of human cells in a chimera is currently low and their distribution throughout the host is random, this experimental setting offers great opportunities. The CRISPR-Cas9 system can be utilized to repress the development of specific organs while blastocyst complementation will provide non-modified iPSCs to rescue organ development. A proof of concept experiment has been carried out on mouse-rat chimera: Pdx1 repression in murine zygote produces apancreatic offspring, whereas rat iPSC injection at blastocyst stage produces mice with the functional pancreas (Kobayashi et al., 2010). This technology, optimized for use in pig-human chimeras, may lead to the creation of excellent transplant incubators. Such chimeric incubators would let pig's developmental program to take control over organ formation as in in vivo approach and negate teratoma risk for patients as in *ex vivo* one.

Reprogramming is currently the only known method that can successfully return adult cells to their ground state. Nevertheless, despite it being a potentially powerful tool for rejuvenation studies, reprogramming is unable to remove all aspects of aging. Even the completely rejuvenated iPSCs retain all somatic mutations obtained by their progenitors. Moreover, emergent forms of aging that consist of upscaled deleterious effects hardly noticeable at subcellular level or artifacts of complex systems' interactions will always be present. For example, imperfect fluid transport within an organism cannot be modified at the cellular level and leads to compound precipitation, local hypoxia, and endocrine response delay. These and other factors would contribute to the deleteriome and ultimately cause organismal aging.

The fight against aging may transcend biology and extend to philosophy. What is the age of an organism whose tissues got replaced? Would it be defined by the irreplaceable parts? If all parts are replaceable, is it possible to stop aging? If we define aging as damage accumulation, aging is not actually stopped when these approaches are used. Damage accumulation occurs spontaneously while its removal needs medical intervention or the development of protective systems (DNA repair, RNA and protein quality control, etc.), which themselves contribute to the deleteriome. In this case, rejuvenation technologies provide only patches that either slow aging by increasing an organism's resistance to damaging factors or replace older structures with the newer ones. Aging, being a spontaneous process, is never really stopped or reversed. Thus, aging is an intrinsic quality of any living system approaching an equilibrium state (death).

Also, aging and age are two different entities: the former is an unstoppable force and the latter is a biological structure's modifiable feature. Despite aging being unstoppable, age can be altered, and epigenetic reprogramming offers a powerful apparatus for this purpose. In this context, aging in biology is akin to gravity in physics (Fig. 3). Never have plane or rocket engineers managed to put together a machine that would defy gravity, but nonetheless this has not stalled progress in aerial transportation. But unlike engineers, biologists are still looking for reliable ways to navigate around their opposing force of nature.

Replacing malfunctioning cells, tissues and organs with their modified copies may prove to be the easiest way to increase human longevity. Although the simplicity of biological manipulation is of high priority when it comes to rewiring the molecular basis of life, the easiest way is not always the most accurate one. Complex systemic factors (such as cellular positioning and inter-systemic crosstalk) should not be ignored to get the truthful notion of aging mechanisms. Multiple studies emphasize a huge yet neglected regenerative potential in humans which can be employed in rejuvenation technologies.

To conclude, aging in biology is akin to gravity in rocket science: a fundamental force that cannot be canceled. Do rockets reverse gravity? No, they do not, but nonetheless they do fly and may escape Earth's grip. Similarly, aging will always have irreversible components, yet that is not a complete roadblock to progress. With a better understanding of biological and deleterious processes, humanity may ultimately manage to navigate through these obstacles to the point when nobody would actually notice that all the printed organs and youth pills exist only

because the aging process can never be stopped or reversed.

Competing interests

The authors declare no conflict of interest.

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