Minireview

Current perspectives on the cellular and molecular features of epigenetic ageing

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Impact statement

The field of epigenetic ageing is relatively new, and the speed of its expansion presents a challenge in keeping abreast with new discoveries and their implications. Several reviews have already addressed the great number of pathologies, health conditions, life-style, and external stressors that are associated with changes to the rate of epigenetic ageing. While these associations highlight and affirm the ability of epigenetic clock to capture biologically meaningful changes associated with age, they do not inform us about the underlying mechanisms. In this very early period since the development of the clock, there have been rather limited experimental research that are aimed at uncovering the mechanism. Hence, the perspective that we proffer is derived from available but nevertheless limited lines of evidence that together provide a seemingly coherent narrative that can be tested. This, we believe would be helpful towards uncovering the workings of the epigenetic clock.

Abstract

It has been noted for quite some time that DNA methylation levels decline with age. The significance of this change remained unknown until it became possible to measure methylation status of specific sites on the DNA. It was observed that while the methylation of some sites does indeed decrease with age, that of others increase or remain unchanged. The application of machine learning methods to these quantitative changes in multiple sites, allowed the generation of a highly accurate estimator of age, called the epigenetic clock. The application of this clock on large human epidemiological data sets revealed that discordance between the predicted (epigenetic age) and chronological age is associated with many age-related pathologies, particularly when the former is greater than the latter. The epigenetic clock clearly captures to some degree, biological features that accompany the ageing process. Despite the ever-increasing scope of pathologies that are found to be associated with accelerated epigenetic ageing, the basic principles that underlie the ticking of the clock remain elusive. Here, we describe the known molecular and cellular attributes of the clock and consider their properties, and proffer opinions as to how they may be connected and what might be the underlying mechanism. Emerging from these considerations is the inescapable view that epigenetic ageing begins from very early moments after the embryonic stem cell stage and continues un-interrupted through the entire life-course. This appears to be a consequence of processes that are necessary for the development of the organism from conception and to maintain it thereafter through homeostasis. Hence, while the speed of ageing can, and is affected by external factors, the essence of the ageing process itself is an integral part of, and the consequence of the development of life.

Keywords: Epigenetics, ageing, DNA methylation

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Introduction

The development of DNA methylation-based age estimators, also called epigenetic clocks, allows for the first time, the measurement of age in all tissues and nucleated cells. Instead of using time, age can now be measured using profiles of biological molecules (methyl groups) that are attached directly to cytosines on the DNA.[1–5] A large body of literature has previously revealed that age has a strong effect on DNA methylation. However, it is only relatively recent that the insight to combine methylation levels of several DNA loci to develop an accurate age estimator was conceived. The resulting "epigenetic clock" is a multivariate predictor of age (or alternatively mortality risk)

based on a multivariate regression model that incorporates methylation values of numerous cytosines. The first epigenetic clock for estimating age based on saliva samples was developed in 2011 by a team from the University of California Los Angeles,[5] which included Steve Horvath, who went on two years later, to present the multi-tissue epigenetic age estimator that applies to all sources of human DNA with the exception of sperm.[1] Other noteworthy estimators include the Hannum clock[2] that is based on 71 CpGs of blood DNA. To date, many other DNA methylation clocks have been developed for different purposes and tissue targets.[3,4,6–18] Since their inception, epigenetic clocks have been used to analyze DNA methylation profiles of diverse human cohorts, uncovering previously unknown associations between accelerated ageing and a great number of health-related features.[19,20] These associations indicate that epigenetic clocks do not just track chronological age, but also biological features that are relevant to the ageing process. Pivotal as they are, epigenetic clocks' association with congenital and non-congenital diseases, lifestyle, and environment are not the focus of this short review. The reader is directed to other reviews that fulfill this purpose.[19–22] What is timely and will be addressed here instead, are the potential molecular and cellular features that underpin epigenetic ageing. This has not been previously reviewed because of the scarcity of available evidence. This shortage notwithstanding, it is time to reassess what is known and to harness clues that could help to better focus attention on future research. We are acutely aware that as we accrue greater depth in our understanding, it is more than likely that modifications to what is described below will become necessary and possibly even an entirely different perspective may emerge. Since insights into mechanisms are found largely through experimental research, these will be the primary source of this article, with occasional references to human epidemiological studies. As such, this opinion piece is not intended to be a catalogue of everything that has been reported to affect epigenetic ageing but a selection of observations with potential connections. The description of the epigenetic clock below will refer largely to the multi-tissue age estimator,[1] unless otherwise stated.

Five methylation features of the epigenetic clock reveal the cellular characteristics of epigenetic ageing

While results from experiments are the bed-rock upon which mechanistic understanding is established in biology, such results are scarce with regard to epigenetic ageing. Despite this lack, much can nevertheless be gleaned through careful deduction from the known physical features of the epigenetic clock. First, methylation of cytosines is undoubtedly a binary event. A cytosine is either methylated or not. As such, a value of 1 is ascribed to a methylated cytosine and 0 to one that is not. Hence, a DNA methylation profile based on 0 s and 1 s can be easily obtained. This, however, is an idealized notion, as almost all CpGs have methylation values between zero and one, such as 0.632. This seemingly unintuitive outcome is a result of DNA samples that are used on Illumina arrays being derived from cellular populations (tissues or cultured *in vitro*) instead of a single cell. Hence, methylation value of 0.632 means that this particular CpG is methylated in 63.2% of cells in the tested population, showing that cell populations, be they from tissues or *in vitro* cell cultures, are epigenetically heterogeneous. The second significant physical feature comes to fore when a comparison is made between methylation values of DNA of those below 35 and those above 55 years of age. The multi-tissue age predictor shows this difference to be on average, 0.032, which translates to 3.2% of the cell population.[1] This suggests that increase in epigenetic age is contributed by changes of methylation profiles in a very small percent of cells in a population.

The third physical feature concerns the rate of methylation change of clock CpGs through the life course. The multi-tissue age predictor shows that this rate is approximately 24 times faster in the first few years of life compared to that after puberty.[1,20] Since changes of methylation constitute the ticking of the epigenetic clock, it means that the clock ticks extremely fast in early post-natal years and much slower after puberty. This pattern is reminiscent of the growth profile of the human body and hints at a connection between epigenetic ageing and the process of development.[23] Indeed, human cohort studies showed that the pace of ticking of the epigenetic clock is associated with more rapid pubertal development in girls[24,25] and greater fat mass and height.[25,26]

The fourth methylation feature of the epigenetic clock is the specific location of the clock CpGs in the genome. From the identity of genes proximal to these CpGs, it was predicted that they would implicate processes involved in cell fate (growth, proliferation, death, survival) and notably, development of tissues and the body. Many of the clock CpGs that become increasingly methylated with age are over-represented near genes whose expression is regulated by polycomb repressive complex (PRC).[1,27–34] Importantly, most PRC-binding sites are bivalent with regard to expression of genes that are proximal to them. This means that these chromatin regions contain histones that promote transcription as well as those that repress gene expression. These bivalent chromatin domains provide the cell with the choice to either activate or repress the genes downstream and hence the plasticity to assume a particular cellular state or identity. If the CpGs of these sites become methylated by Dnmt3a/b, the expression of the downstream genes would be committed to a single state and the cell loses its potential or plasticity with regard to its identity.[35] Hence, PRCs compete with Dnmt3a/b for binding to the same target. With age, for reasons yet to be determined, these PRC targets become methylated in increasing number of cells in a population. The link of PRC with development is especially clear since it is a repressor of HOX genes, which specifies segmentation of the embryo along the head to tail axis of the organism.[36] It controls the expression of genes necessary for embryonic development, stem cell differentiation, and tissue homeostasis. Proteins that constitute the PRC are conserved across a large swathe of plant and animal species, ranging from flies to vertebrates;

signaling their primeval credentials. Significantly, the PRC and its associated proteins regulate longevity of flies.[37] It is worth appreciating the fact that the epigenetic clock is constituted in part by CpGs that are associated with a truly ancient mechanism that regulates development. While the underlying mechanisms remain to be fully understood, the association between ageing and the process of development and tissue homeostasis is very difficult to ignore.

The above association between clock CpGs, PRC targets, and development provides a smooth lead into the fifth feature of the epigenetic clock, which is its applicability to prenatal biological samples and embryonic stem cells. The multi-tissue epigenetic clock is able to recognize and correctly predict ages of fetal tissues[38] and that of embryonic stem cells (negative age, approximately -0.5 years).[1] This is profound as it demonstrates that the epigenetic clock, which captures at least some degree of biological ageing, begins ticking very early on before birth. The implications of this are two-fold. The first is that biological processes that constitute the ticking of the epigenetic clock, whatever they may be, are already in motion very soon after conception. The second is that since the processes associated with ageing already begin before birth, it introduces the notion that the ageing process is an integral part of, and the consequence of the development of life.

Collectively, these five features of DNA methylation allow one to summarize with some degree of certainty that epigenetic ageing is a measure of change of epigenetic heterogeneity, contributed by a relatively small percentage of cells, seemingly in line with developmental processes that are conserved across species and begins very soon after conception. This seemingly inescapable deduction provides us with a reference point against which models and hypotheses can be measured. While consistency with all the five attributes does not guarantee veracity of a model, inconsistency with any one, will signal the unlikely validity of a hypothesis.

Cellular mechanisms

Cellular senescence

The above characterization of the cellular components of epigenetic ageing ushers in the intuitive suspicion that these cells may be senescent cells. After all, senescent cells increase in number with age, are involved in developmental processes[39] and are undoubtedly mediators of ageing characteristics through secretion of harmful and pro-inflammatory molecules.[40–44] Consistent with this, removal of senescent cells reverse age-related decline of tissue function.[45] As such, it appears likely that DNA methylation profile of senescent cells, could constitute the ticking of the epigenetic clock. Indeed, one of the early experiments appeared on the surface, to support this notion. It was observed that cellular populations that become senescent through exhaustive proliferation (replicative senescence) exhibited increased epigenetic age.[46] This, however, was not the case with radiation-induced senescence and oncogene-induced senescence. This could of course be due to the distinctiveness between different types of senescent cells. Alternatively, the increased epigenetic age that accompanied replicative senescence could be owed to the extended period of time with which they had to be in culture before becoming senescent, during which they underwent epigenetic aging. This uncertainty was addressed by preventing primary cells from replicative senescence by transducing them with telomerase, which maintains telomere length. This did indeed prevent replicative senescence, but it did not prevent epigenetic ageing, which continued unabated.[3,47–49] These repeated observations clearly indicate that while telomere attrition and senescent cells are undoubtedly important features of ageing, they do not constitute the epigenetic clock.[50,51] Consolidating this conclusion is the multiple independent demonstrations that DNA methylation changes that accompany cellular senescence are distinct from those of epigenetic ageing.[52–54]

Cellular proliferation and terminal differentiation

Thus far, neonatal keratinocytes, dermal fibroblast, adult coronary endothelial cells, and fetal retina have all been shown to undergo epigenetic ageing in culture.[3,47,49,55,56] These different cell systems allowed the testing of another intuitive assumption, which is that epigenetic ageing may be related to cellular proliferation. This notion, however, was swiftly discounted when it was demonstrated that keratinocytes that underwent greater rate of proliferation and exhibited extended proliferative capacity had similar rates of epigenetic ageing as their isogenic control.[55] Similar absence of correlation between proliferation rate and epigenetic ageing rate was also seen with fibroblasts[49] and gut epithelial cells.[57] These observations are entirely consistent with the fact that tissues with very different turn-over rates such as skin, blood, and bone, from the same body, exhibit similar or comparable epigenetic age;[1,3] negating the notion that the pace of epigenetic ageing is either a measure of, or determined by cellular proliferation. Furthermore, somatic cell differentiation such as terminal differentiation of keratinocytes[55] or gut epithelium[57] also does not alter the rate of epigenetic ageing. Likewise, fetal retina which differentiates and develops in culture continues to undergo epigenetic ageing.[38] Collectively, these reports show that epigenetic ageing is neither a measure of cellular proliferation nor that of cellular differentiation.

The exception to the rule

As listed above, many different human primary cells undergo epigenetic ageing in culture. This feature, however, does not extend to embryonic stem cells. Extensive passaging of stem cells up to even a hundred times is not accompanied by increase in epigenetic age.[1] Interestingly, the epigenetic age of embryonic stem cells is below zero, i.e. pre-natal, which indeed it is. Although the epigenetic clocks of embryonic stem cells do not tick regardless of how many times they proliferate, epigenetic ageing clearly occurs *in utero*; during gestation.[14,58–60] Hence, the ticking of the epigenetic clock must be initiated soon after differentiation of the embryonic stem cells. Indeed, we posit that

it may start as soon as the cells exit from the embryonic stem cell state.

It is worth digressing a little at this point to consider an important implication of this notion. An intuitive assumption of ageing is that it is not an intrinsic property of life, but an extrinsically induced feature, which is encapsulated in the term "wear and tear". The fact that epigenetic ageing clock starts ticking very soon after the embryonic stem cell stage, prompts a re-assessment of this perspective. The evidence suggest that processes related to ageing are already afoot at the start of life. The involvement of DNA methylation changes to specific CpGs indicates that this is a very precise process that is inconsistent with entropy or randomness, which is the nature of damage. It is important to consider, however, that these changes are meant to develop the organism. Hence, the ageing process is a continuum which begins after the embryonic stem cells state through to death. Therefore, the essence of ageing might well lie in understanding why developmental processes inadvertently contribute to ageing of the organism. It is important to note that although wear and tear or damage-related events appear not to be the "prime-mover" of epigenetic ageing, they do nevertheless affect the rate of epigenetic ageing.[61,62]

Another important point when considering the potential involvement of stem cells in epigenetic ageing pertains to reprogramming of adult cells into the pluripotent state—induced pluripotent stem cells (iPS). While such reprogramming by Yamanaka factors are well attested,[63] the age of the reprogrammed cells was less so, although it was assumed that having reverted to the pluripotent state, they would naturally be young. This, however, was not necessarily a foregone conclusion as it is possible that reprogrammed cells might retain the age they were at when they were re-programmed. In such a case, they would be "old stem cells" and this would uncouple the process of development from epigenetic ageing. As it happens, re-programming does indeed reset the epigenetic clock to prenatal age,[1,64,65] signifying rejuvenation. This strengthens the point that epigenetic ageing is a "read-out" of cellular processes that initiate from the start of life that are necessary for development of the body, and when this developmental state is reversed, so does epigenetic age.

Ticking of the clock and organism development

Having ascertain when the epigenetic clock starts ticking, we can now consider what constitutes its ticking. A cell type that fulfills the five criteria described above is tissue stem cells or progenitor cells—(a) being a part of the heterogenous mix of cells within a tissue, (b) being a very small proportion of cells within a tissue, (c) undergoing changes that drive the process of development, (d) having PRC regulate their differentiation, and (e) are present and functioning from the very early stages of embryonic development. For the sake of descriptive simplicity and the current scarcity of information, when stem cells are mentioned in the description below, it could as well potentially apply to progenitor cells. To consider the potential means by which tissue stem cells may be involved in the ticking of the clock, it is worth re-assessing the known features of these cells. Stem cells can undergo symmetric or asymmetric proliferation. The former will result in two stem cells, while the latter will also produce two cells, but one of which will retain the stemness of the mother cell, while the other will become a non-stem cell, which for the sake of simplicity can be referred to as transit-amplifying cells. It is thought that stem cells proliferate infrequently, and the bulk of proliferation activity of tissues is performed by transit-amplifying cells. One could posit that the ticking of the clock is a measure of asymmetric stem cell division, i.e. when one of the daughter cells become a transit-amplifying cell, its DNA methylation profile changes and this is manifested as alteration of the methylation profile of the tissue—the ticking of the clock. As stem cells are in low abundance and undergo infrequent division, the DNA methylation changes of tissues that occur in time would be very small, which is indeed observed for the ticking of the epigenetic clock. This scenario would suggest that were stem cells to be isolated from tissues (free of transit-amplifying cells and fully differentiated cells) and analyzed, their DNA methylation profiles would indicate much younger epigenetic age than the tissue. This was indeed observed in a recent report showing that muscle stem cells isolated from mice were epigenetically much younger independently of the ages of the tissue/animal from which they were derived.[66] Likewise, small intestine and colon crypts, in which stem cells reside have younger epigenetic age than the upper portion of the tissue which are devoid of stem cells.[57] Although these do not by themselves constitute proof, it is nevertheless consistent with the notion that ticking of the epigenetic clock is constituted by methylation changes that accompany the differentiation of stem cells into non-stem cells (such as transit-amplifying cells). Importantly, this proposition is testable with further experiments analyzing stem cells of different tissue and organs, and there is little doubt that results from such experiments will become available in the very near future. It is however important to note that the hypothesis proffered is based on our current understanding of cell types and identities in tissues. We do not presume this understanding to be comprehensive or highly accurate. As such, it is necessary to entertain the possibility that instead of stem or progenitor cells, a yet-to-be-identified cell type within tissues is responsible. Also, instead of changing their states (such as differentiation), the stem cells or yet-to-be-identified population of cells may contribute to age-related methylation changes by either gradually increasing or decreasing their number in tissues. There are many more hypotheses that can be proposed, but they are predicated on even more layers of assumptions, each of which would confer increasing degree of uncertainty to the hypotheses, and most of which cannot be easily tested. Hence, what is described above is necessarily a conservative proposal that is in keeping with what is currently known and adhering to the five attributes of the epigenetic clock, with minimal assumptions and most importantly, is readily testable.

Ticking rate of the clock and organism maintenance

Having considered what might constitute the ticking of the clock, we can proceed to address the rate of ticking. After all, it is not the ticking of the clock that is associated with increased risk of age-related pathologies but the increased ticking rate that is thus so. To this end, it is important to consider the biology beyond development and across the entire life-course. From the blastocyst stage onwards, stem cells undergo a very high rate of differentiation to generate specialized cells to build and develop tissues. This rate would eventually reduce as the required cell number and maturity are attained and organs are fully formed. This is indeed reflected in the different rates of methylation changes to the clock CpGs in function of age.[1] From the state of maturity onwards, however, differentiation of stem cells would serve to maintain the body by replenishing tissues with fresh cells to replace dead ones, to establish homeostasis. Evidently, tissues have different turn-over rates with some such as the skin and the gut, having particularly high ones. It would stand to reason that turn-over rates of tissues would reflect differentiation rates of their stem cells into non-stem cells (or transit-amplifying cells) and hence also their epigenetic ageing rates. As such, it is perceivable that tissues with very different development and maintenance dynamics may have epigenetic ages that are significantly divergent from the other tissues. In this regard, it would be informative to consider in the sections below, the tissues such as the brain—the cerebellum in particular, the female breast, the muscle, and the retina; all of which exhibit poor concordance with epigenetic age of other tissues.[38,67]

It was previously thought that the human brain does not undergo any cellular turn-over after full development and maturation. Even now, adult neurogenesis is thought by some to not occur at all. While direct human experimentation to address this is associated with unsurmountable challenges, the extrapolation of observations from animal experimentation to the human brain is not without limitations and uncertainties. A solution to this impasse comes from an unexpected source—the Bomb Pulse.[68] This is a pulse of carbon-14 (C14) that was released into the earth's atmosphere as result of nuclear bomb testing which started in earnest from 1945 leading to a peak between 1950 and 1963, after which above-ground nuclear bomb test was banned. The elevated levels of C-14 in the atmosphere began to decline steadily, at about 4% a year and will return to pre-bomb test level by 2033. During this period, this pulse of C-14 was incorporated into all life-forms on earth, including humans. In accordance with the combined principles of carbon-dating and pulse-chase experiments, the incorporated C-14 in biological materials allows the determination of the age of cells, tissues, and organisms. Spalding *et al.*[69] employed this method to address the question of adult neurogenesis and observed that it does indeed occur but the turn-over of cerebellum is significantly lower than that of the occipital cortex and possibly the lowest of the body. This is consistent with the epigenetic age of cerebellum, which is much lower than that of the other parts of the brain and body.[67] While this does not constitute proof, it does bear serious consideration as two very different technical measurement produced results that are consistent with each other; namely tissue turn-over rate is reflective of epigenetic ageing rate.

Another developmental feature linked with epigenetic ageing is that of the female breast, which exhibits a very significant augmentation of age compared to that of blood, which is generally in good agreement with chronological age.[1,70,71] This disparity is consistent with and mirrors female breast development. While most tissues cease development and switch to maintenance soon after puberty, female breast tissues continue to develop pass this stage, underlining the parallel between developmental processes and epigenetic ageing.

The opposite situation appears to occur in muscles. The proliferation and differentiation of muscle stem cells cease upon physical maturation. These activities are initiated in adult muscles only in response to injury. Consistent with the proposed model, the epigenetic age of muscle cells is found to be younger than the rest of the body.[3] Finally, a further example concerns the retina. During retinal development in the human fetus, the prenatal epigenetic age closely mirrors the chronological age.[38] This ceases soon after the retina is fully developed, and there is almost no turn-over of the tissue. Consistent with this, the rate of epigenetic ageing slows down very significantly, and chronological age, by its nature continues, leading to the loss of concordance between the two types of ages.

While the indications are strong that stem cell differentiation in the context of tissue development and homeostasis underlie epigenetic ageing, direct empirical evidence is still scarce. We would like to highlight a potential weakness of this hypothesis, which is that it does not address the fact that the number of hematopoietic stem cells increases with age, albeit with vastly reduced function,[72–75] while those of other tissues, such as muscle stem cells appear to decrease with age.[76–79] Whether this discrepancy is of significance is presently unclear. Hence, the above-mentioned involvement of a yet-to-be-identified cell type as being responsible for epigenetic ageing instead, must for now remain a viable alternative hypothesis. Validation or rejection of this notion requires *in vivo* experimentation with animal models, for which gratifyingly epigenetic clocks are now available.[22,64,65,80–84]

Molecular mechanisms

The most intuitive approach to elucidate the molecular mechanisms that underpin the epigenetic clock is to identify the genes whose expression is altered by the clock CpGs. This seemingly straightforward approach runs into several considerable challenges. First, only 10% of the multi-tissue clock CpGs are located in promoter/enhancers, while the rest are not.[1] It is not a foregone conclusion that only the former will affect gene expression or that the latter will not. It is equally uncertain that genes that are most proximal to the clock CpGs are necessarily regulated by them. Genes at a distance might instead be the target of such regulation. Second, the average lifespan change in DNA methylation is very small indeed.

Hence, the transcriptional consequences of these methylation changes will most likely be manifested by a miniscule number of cells and these are readily masked by the transcripts of overwhelming majority of cells. It is evident that single-cell RNA sequencing is required to identify these changes in a very small percentage of cells. As of the time of writing, there is no publication known to us, where this approach has been applied to address this question. No doubt these will be available in the very near future.

In the meantime, the availability of an *in vitro* epigenetic ageing cell culture system has opened the way to the testing of compounds, conditions, and modalities that can affect the rate of epigenetic ageing and thus hint at its nature and possibly the molecular pathways that are implicated.[47,55] Already, the effects of telomere maintenance, cellular senescence, DNA damage signaling, terminal differentiation and cellular proliferation have all been tested and found to be unrelated to epigenetic ageing.[47,55] On the other hand, inhibition of mTOR by rapamycin, caloric restriction, hypoxia, and growth hormone appears to do so.[49,55,64,80,81,85,86]

mTOR and caloric restriction

The implication of mTOR is particularly interesting because genome-wide search for genes that are associated with accelerated ageing uncovered a SNP within the MLST8 gene as that with the highest hit.[87] Interestingly, the expression of this gene increases with age and its protein product, MLST8 stimulates the formation of the mTOR complex,[88,89] indicating an age-related increase of mTOR activity. Significantly, rapamycin, which inhibits mTOR activity retards epigenetic ageing *in vitro*[55] and *in vivo*.[81,85] The consistency of the conclusions drawn from genome-wide studies and two very different routes of investigations (*in vivo* and *in vitro*) is very compelling and strengthens the notion that an mTOR-mediated molecular pathway is at least one of the mechanisms that underpins epigenetic ageing. While this is an important step forward, it is merely the start as mTOR is implicated in a great number of activities including nutrient sensing, cell growth, cell cycle, cellular senescence, cell survival, DNA repair, amino acid imbalance, energy regulation, autophagy, and cell survival.[90–92] While we await further detailed investigations to tease out the activities relevant to epigenetic ageing, it is worth noting that perturbation of the nutrient sensing pathway through caloric restriction is currently the most effective intervention with regard to increasing life-span with reduced morbidity across multiple animal models.[93,94] Indeed, caloric restriction slows epigenetic ageing of mice.[64,65,81,82,86] In line with this, there is some suggestive evidence that glucose levels may increase the rate of epigenetic ageing.[56]

Oxygen effect

Oxygen levels appear to affect the rate of epigenetic ageing, with increasing rate accompanying the increased levels of oxygen.[49] Importantly, this oxygen effect occurs without any alterations to cellular proliferation; once again demonstrating that epigenetic ageing rate is not determined by proliferation. The mechanism underpinning this remains to be elucidated but hypoxia has been demonstrated to inhibit mTOR activity.[95–97] Hence, the slowing down of epigenetic ageing in hypoxic condition may function in a similar way as rapamycin by inhibiting mTOR activity and slowing epigenetic ageing. We acknowledge that this is an overtly mTOR-centric view and that other explanations are possible. For example, this effect could instead be mediated by HIF1a, a protein whose level increases in hypoxic state. HIF1a has been reported to repress the TET2 protein which catalyzes demethylation of DNA,[98] while paradoxically, TET1 protein is reportedly able to stabilize HIF1a[99] instead. Compounding this complexity is the report that HIF1a stimulates DNA methylase activity.[100] It is readily conceivable that in principle, such changes to enzymes involved in DNA methylation can impact epigenetic ageing, but it is far from clear whether this is indeed the case as the potential involvement of HIF proteins in DNA methylation is convoluted[101] and at this stage there is no immediately obvious mechanism that could be envisaged.

Circadian clock

The above-described age-accelerating effect of high oxygen level may impose an hitherto unappreciated impact on epigenetic ageing rates of primary cells (human keratinocytes, dermal fibroblasts and coronary artery endothelial cells) in culture.[47,55,56] It appears that while cells isolated from tissues are still fully capable of undergoing epigenetic ageing, their rates are significantly higher compared to when they are *in vivo*. It would appear that when primary cells are isolated from tissues and put in culture, they become unshackled from the mechanism that regulates their ageing rates in the body. This free-running *in vitro* state may be compounded by the effects of unusually high oxygen content of *in vitro* culture condition. The description of a "free-running" state in context of epigenetic ageing bears significant parallels with the circadian rhythm.[102] Both the epigenetic and circadian clocks are present in all cells of the body, but their ticking rates are regulated. The circadian clocks of all cells are synchronized by the suprachiasmatic nucleus (SCN) through endocrine and nervous cues. In turn, the SCN is regulated by light-induced retinal-ganglion signals. While the underlying mechanism of synchronicity of the epigenetic clocks between tissues has yet to be fully elucidated, both these clocks lose synchronicity when cells are isolated from tissues and grown *in vitro*. These similarities compel one to ponder potential links between them. In this regard, it may be of significance that Bmal-1, a transcription factor that regulates expression of circadian genes, represses mTOR signaling and retards ageing.[103] At the same time, mTOR regulates central and peripheral circadian rhythms,[104,105] which is also regulated by caloric restriction.[106] Whether these mutual interactions feed into epigenetic ageing or vice versa is not a foregone conclusion and is ripe for investigation. Once again, potential links to mTOR are evident but these should not exclude consideration of other possible mechanisms.

Rejuvenation

Recently, it was reported that administration of human growth hormone, metformin and dehydroepiandrosterone (DHEA), which were intended to regenerate the thymus, reversed epigenetic ageing of nine men.[107] This is undoubtedly an exciting development as it suggests that appropriate interventions may not only retard or halt ageing but even reverse it. It is not known at this stage whether the rejuvenating effect is mediated through the regeneration of the thymus or a direct effect of the treatment modality on the body. Also, it is not known if the effect is mediated by all three compounds or one or two of them. It is of interest to note that metformin has previously been reported to retard several ageing phenotypes and extend lifespan in various animal model.[108–111] Hence, it may be a critical ingredient in the mix, although its effect on epigenetic ageing was not detected in observational studies.[112] The role of growth hormones is complex. On the one hand, a substantial literature reports pro-ageing and life-shortening effects of growth hormones in mice.[64,65,113,114] This would appear to suggest that human growth hormone may not be the active compound. On the other hand, short-term growth hormone interventions (12 months) are beneficial when administered to older men (aged 50–65). The seemingly opposing properties of growth hormones bring to fore the saying of Paracelsus, that dose and timing make the poison. It is also worth noting that there may be significant differences between mice and humans with regard to the effects of hormones on ageing. On the other hand, it is notable that there is similar inter-species response with regard to estrogen. Menopause in women is accompanied by increased epigenetic age, which is also the outcome of removal of ovaries of mice.[80,115] Clearly, what we know at this stage does not allow the formation of general principles regarding the impact of hormones on epigenetic age, but their involvement in development and maintenance of the body argue that they do indeed have a very significant impact on the epigenetic clock.

Conclusion

This short opinion piece is limited to salient features of DNA methylation-based biomarkers of ageing that relate to the inner workings of the epigenetic clock at the cellular and molecular level. There is no doubt that in this relatively early period since the development of the epigenetic clock, solid evidence is scarce. This notwithstanding, the information that could be harnessed from the methylation features of the clock is substantial and they point to a link between epigenetic ageing, stem cells, and processes of development and maintenance. The molecular features of the clock remain to be elucidated, but it is likely that nutrient sensing pathways play an important role. How the molecular features fit with the cellular features will be the focus of future research. No doubt, the recent availability of epigenetic clocks for animals will greatly help in this endeavour.[64,65,80–84]

Regardless of the underlying mechanisms, it is difficult to ignore the fact that epigenetic ageing begins from very early moments after the embryonic stem cell stage and continues un-interrupted through the entire life-span. The significance of this is profound as the question of why we age has been attributed to many different things, most commonly to "wear-and-tear." The ticking of the epigenetic clock from the embryonic state challenges this perspective and supports the notion that ageing is an unintended consequence of processes that are necessary for the development of the organism and tissue homeostasis thereafter. It is important to note that this does not negate the important contribution of other features of ageing such as telomere attrition, DNA damage, and cellular senescence, which affect the rate of biological ageing as a whole. Instead it highlights the important point that the effectiveness of age-mitigation measures such as those against senescent cells or towards telomere maintenance may not be effective against epigenetic ageing. Hence, it is important that the molecular and cellular mechanisms underpinning epigenetic ageing be elucidated to reveal potential points of intervention.

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