ISSN 0006-2979, Biochemistry (Moscow), 2013, Vol. 78, No. 9, pp. 1048-1053. © Pleiades Publishing, Ltd., 2013.
Published in Russian in Biokhimiya, 2013, Vol. 78, No. 9, pp. 1337-1344.

How Does the Body Know How Old It Is? Introducing the Epigenetic Clock Hypothesis

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Received February 15, 2013

Abstract—Animals and plants have biological clocks that help to regulate circadian cycles, seasonal rhythms, growth, development, and sexual maturity. It is reasonable to suspect that the timing of senescence is also influenced by one or more biological clocks. Evolutionary reasoning first articulated by G. Williams suggests that multiple, redundant clocks might influence organismal aging. Some aging clocks that have been proposed include the suprachiasmatic nucleus, the hypothalamus, involution of the thymus, and cellular senescence. Cellular senescence, mediated by telomere attrition, is in a class by itself, having recently been validated as a primary regulator of aging. Gene expression is known to change in characteristic ways with age, and in particular DNA methylation changes in age-related ways. Herein, I propose a new candidate for an aging clock, based on epigenetics and the state of chromosome methylation, particularly in stem cells. If validated, this mechanism would present a challenging target for medical intervention.

DOI: 10.1134/S0006297913090113

Key words: biological clock, senescence, rhythm, maturation, aging, programmed aging, adaptive aging, methylation, epigenetics, gene expression

There are many indications that aging is a genetic program in most higher organisms including humans (reviewed by Mitteldorf [1-3]).

• Many of the genes that regulate aging have been conserved since the dawn of eukaryotic life [4]. All other such highly conserved genes have been protected by natural selection because they form an essential core to life processes. Natural selection has evidently treated aging as a core life process.

• Hormesis: The fact that life span can be readily extended under genetic control when conditions are most harsh and challenging (e.g. starvation) indicates that the body is "holding back" on life span at times when the environment is more favorable [5, 6].

• Breeding animals for longevity does not necessarily impair fertility as is demanded by popular theories based on pleiotropy or trade-offs [7]. In fact, many single-gene mutations are known to extend life (especially in worms), for which no major cost has yet been identified [8].

• One-celled eukaryotes are subject to two modes of programmed death: apoptosis [9] and replicative senescence [10-12]. This fact in itself vitiates the classical theoretical contention that it is impossible for programmed death to evolve, requiring as it would an implausible triumph of group selection over individual selection. Both processes and their function have been conserved, so that they continue to play a role in the aging of higher organisms, including humans [13, 14].

Accepting the premise that aging is programmed, we might ask about the mechanisms by which the process of senescence is directed and organized. How does the body measure time? How does the body maintain a record of how old it is? Does the existence of an aging program imply that there is a master clock?

If a program for aging were designed by a human engineer, it would be based on a central (flexible) time-keeping mechanism; but this is not necessarily the system that natural selection has bequeathed us. In particular, there is long-term group selection in favor of aging, but strong short-term individual selection against it. In order to shield affirmative aging mechanisms from dismantlement at the hand of individual selection, it is likely that evolution has embedded them below the surface, and deployed redundant time-keeping [3, 15]. A single master clock would be easily hijacked by individual selection. We might expect to find several interdependent and redundant clocks for aging.

Under the paradigm of programmed aging, senescence may be a continuation of development, and we

might suspect that whatever controls the timing of development has been extended to regulate the timing of aging. But neither a developmental clock nor an aging clock has yet been discovered. The closest thing we know of is cellular senescence, based on telomere length [16]. But there are many organisms that age, in which telomerase is freely expressed, telomeres remain long, and thus the telomere clock is not operating; also, it is unlikely that the telomere clock would control the timing of growth and development. These considerations make it more likely that there is another clock and that it is somehow "hidden in the works" of metabolism, so that it would not have been obvious to investigators thus far.

My hypothesis, proposed below, is that gene expression itself forms a kind of aging clock. Time is maintained within the signal networks of metabolism, and a running record of the organism's age is imprinted in the methylation state of the genome. Gene expression products are part of a signal cascade that affects all aspects of metabolism, but that also feeds back through methyl transferases to increment the clock.

Below, I will first briefly survey known biological clocks, including aging clocks, and discuss prospects for medical interventions that might manipulate them. These include thymic involution, the suprachiasmatic nucleus, the hypothalamus, and replicative senescence. The latter, based on telomere attrition, has been the subject of intense research in the past decade, with promising developments ongoing. I discuss interventions based on telomere clocks elsewhere in this issue [16]. Finally, I argue based on evidence and logic that the methylation state of the genome, within stem cells in particular, may be a promising place to look for a stored record of organismic age that informs the body's growth, development, and senescence.

AGING CLOCKS

A clock has both a motor and a dial. It not only measures the passage of time, but also stores reference information about what time it is now. Biological systems also both measure the passage of time and keep a record of the current age. We know this because even if the metabolism is transiently disrupted, the body remembers how old it is and restores to a homeostatic condition that reflects its current age. Yeast cells can be frozen, and remember their age when thawed. Starfish are a dramatic example: a starfish can be dismembered and regenerated from a piece of itself, and the individual that regrows has a remaining life span that depends upon the age of the original animal from which it was cloned.

In a clock, the motor and escapement or pendulum are more complicated than the clock dial, and we might expect the same thing in biology. Even though the information that is processed to determine the rate of aging is diverse and complex, dependent through neuroendocrine processing on multiple internal and environmental factors, nevertheless we might reasonably hope that the clock dial itself is considerably simpler.

The worst case would be if the body only subjects itself to damage and dysregulation with age. The information about age could conceivably be stored globally as damaged proteins and mutated DNA and wrinkled skin and decalcified bones, etc. This is a possibility that has been raised by Aubrey de Grey (personal communication), and it would not bode well for anti-aging science. In this scenario, it may be that aging is programmed, but the nature of the program offers no opportunities for simple, upstream interventions that slow aging or set back the clock. We are left with the challenge of engineering a repair for diverse forms of damage, just as if aging had been a disordered accumulation of damage.

But there are several good indications that this pessimistic scenario is not the case, and in fact there is a relatively simple, localized "clock dial", with hands that we might hope to reset to an earlier age.

- Aging responds to signals (e.g. mTOR, SIRT1, FOXO, IGF1) in a powerful way that would not be possible if there was only the state of damage to mark the passage of time.
- Some interventions are known that successfully roll back the clock in model animals. Adult carrion beetles [17] can be manipulated with starvation to regress back to a larval state, and they can cycle multiple times through their life history. *Turritopsis* has been known to do this in the wild [18, 19]. Lab mice have been rejuvenated with telomerase [20, 21] and with blood factors [22, 23].
- Finally, though we may not know the mechanism (besides state of damage) by which the body stores information about age, we can be confident that there is one, because it is absolutely essential for development. The body needs to know when to grow, when to stop growing, when to begin expressing sex hormones, triggering the onset of fertility. It is not reasonable to think that these events are triggered by a state of accumulated damage or by telomere length. Whatever clock mechanism is responsible for the timing of development and puberty is capable in theory of continuing its operation for the purpose of timing senescence.

Almost all the aging interventions that have been explored experimentally to date manipulate the clock *mechanism* but not the *dial*, affecting the rate of aging but not directly the present age. The exception appears to be in telomerase activation [20, 21, 24]. This constitutes evidence that replicative senescence is one clock affecting human aging, and telomere length is its clock dial. Another possible exception, less well understood, was found for drosophila flies placed on caloric restriction (CR) in mid-life. Promptly upon application of CR, their mortality rate drops to a rate characteristic of an earlier age [25].

KNOWN BIOLOGICAL CLOCKS

Circadian biological clocks have been widely studied and are partially understood. There is evidence for an annual clock that contributes, along with environmental cues, to patterns of migration and hibernation; but mechanisms have not been identified. And the timing of growth, reproductive maturity, and senescence remain still more mysterious.

The mammalian circadian rhythm is known to be regulated from the suprachiasmatic nucleus, a small structure in the middle of the brain [26]. A chemical mechanism (based on peroxiredoxins) has recently been discovered that might underlie all circadian clocks [27]. The cycle is responsive to light and dark, and the intrinsic period is close enough to 24 h that circadian timing becomes entrained with diurnal cycles.

It has been proposed that seasonal cycles in mammals are mediated through a response to light in the pineal gland. The mechanism is thought to be independent of the circadian clock [28]. Again, there is an intrinsic cycle time that is modulated by temperature and duration of daylight.

In female mammals, onset of puberty is controlled by a single chemical signal: gonadotropin-releasing hormone (GnRH). But the timing of this trigger is controlled in turn by a complex calculation, based in neural as well as hormonal mechanisms. "The anatomical development of the GnRH secretory system occurs relatively early in life, and that the synthetic capacity is present well before puberty in that GnRH mRNA expression reaches adult levels" [29]. Timing responds to olfactory cues, stress, fat reserve, activity, season of the year, and other stimuli. The workings of this clock remain mysterious.

Aging responds to these same cues, and perhaps to others. There is reason to believe that the aging clock mechanism is at least as complex as the developmental clock. Though aging is programmed, it may not be programmed in a simple way. This accounts for the challenge that aging has posed for research and medical intervention. The fact that aging progresses under genetic control suggests a promising approach to anti-aging interventions, and yet the complexity of the timing mechanism has slowed the pace of progress.

Although relationships between the circadian clock and the aging clock have been documented, these are not such as to suggest that the aging clock depends directly on a count of circadian cycles. For example, dysregulation of the circadian clock *in either direction* leads to accelerated aging in flies [30, 31].

The Neuroendocrine Theory of Aging was proposed by Vladimir Dilman in 1954 [32]. Homeostatic control of hormone secretions is supported by the hypothalamus, and different hormonal levels are maintained as appropriate for different stages of growth and development. Dilman's hypothesis was that the trajectory of changes in the hypothalamus has a kind of momentum ("hyperadaptosis") that carries forward after maturity and results in "dysregulation" that characterizes the aging phenotype. The Neuroendocrine Theory is an early precedent for the Epigenetic Theory described below. "The life span, as one of the cyclic body functions regulated by "biological clocks", would undergo a continuum of sequential stages driven by nervous and endocrine signals" [33]. But Dilman did not frame this theory within the context of an adaptive program shaped by natural selection, and therefore the concept of a biological clock sits uncomfortably within its narrative.

The Immunologic Theory was proposed by Roy Walford in 1962 [34, 35]. The proliferation of immune cells in the blood constitutes a kind of clock, which becomes dysfunctional as the number of cells multiply. The body's cells are mutating as the number of different immune memory cells is multiplying. Chance coincidences result in the immune system attacking self with increasing frequency over time. Walford noted how many diseases of old age have a relationship to autoimmunity, but never connected this to programmed aging. He saw thymic involution as an independent cause of immune failure, and perhaps another aging clock.

The hypothesis that cellular senescence represents a primary aging clock was promoted by West, culminating in a popular book published 2003 [36]. That same year saw Cawthon's actuarial study, associating leucocyte telomere length with mortality in humans. In the years since then, evidence has accumulated for the importance of telomere length in human aging, and several herbal extracts that are claimed to address it have reached the market. In a companion article in this issue, I discuss the telomeric clock in greater detail [16].

EPIGENETIC CLOCK HYPOTHESIS

In the fall of 2012, an article [37] appeared by Adiv Johnson and a diverse team of scientists from the US and Europe pulling together evidence that the methylation state of the genome is related to the body's age, and proposing methylation as an appropriate target for anti-aging research. I would extend their proposal to argue that, if we believe there is an aging clock, the methylation state of the genome (especially in stem cells, because of their persistence) is logically the first place to look for its "clock dial". Seeking a system of global signals that affects the metabolic state of the entire body, we would look as far upstream as possible. Upstream takes us to gene expression. Further up, there may be signals that affect gene expression globally, but these, too, are products of genes, and hence they can be regarded as part of a self-modifying program for gene expression. If there is not in evidence another, separate clock which feeds down to affect gene expression, then it is logical to assume that this self-modifying program functions as a clock in its own right.

We know that gene expression changes with age, and that this has the potential to affect all aspects of the metabolism and the aging phenotype. If there is an aging clock, then its output must be transduced so as to affect gene transcription. Merging the clock into the transcription state of the genome would be the most economical implementation of a clock mechanism, obviating the need for a separate record of the age state of the body. Gene transcription is affected by transient signaling, and also by more persistent epigenetic markers. The most important of these persistent markers is the genome's methylation pattern. The "methylome" contains information that is both programmable and persistent. Cytosine (the "C" in ACGT) is one of the four nucleic acid residues that form chromosomal DNA. Within the DNA molecule, cytosine can accept a methyl group to form 5-methylcytosine, and this suppresses transcription locally in genes where methylation has occurred [38]. Methylation patterns tend to be copied along with DNA replication, and they can even last through several generations as a form of epigenetic inheritance [39].

An epigenetic clock has the potential to regulate growth, development and sexual maturity, as well as aging. If no other clock has been discovered that controls the timing both of development and aging, then our default hypothesis ought to be that the epigenetic state of the genome is its own clock.

The methylation state of the genome is also self-modifying in the sense that transcription of methyl transferases and related enzymes creates the mechanism for feeding back upon the methylation state. This feedback implies the basis for a clock mechanism. Genes that are transcribed today create the metabolic environment that cascades into signals that reconfigure the methylation state and program the genes that will be transcribed tomorrow.

The above constitutes a general, theoretical argument for epigenetic state as an aging clock. There are also specific experimental results that point in this direction.

• Gene expression profiles change substantially with age. There is reason to believe that an individual with youthful gene expression is functionally a youthful individual [37].

• Methylation has about the right degree of persistence. We know that methylation contains epigenetic information that is passed on in a soft way when DNA is replicated.

• In general, methylation decreases with age (though there are characteristic regions that become hypermethylated). Hypomethylation has been associated with "frailty" and markers of biological age [40].

• Fruit flies with a copy of the methyl transferase *dnmt2* in their genome lived 58% longer than control flies. Conversely, flies engineered to be +/– for *dnmt2* had life spans 25% shorter [41].

• Similar experiments with mice yield more nuanced results. Early, studies that have not been reproduced reported that methylation was actually higher in *dnmt1* +/– mice than in +/+ controls [42, 43]. More recently, neural deficiencies and low bone densities, increasing with age, have been reported associated with engineered *dnmt1* deficiencies [44].

• In monozygotic twins, methylation patterns are similar when young, but diverge over time [45]. This suggests a stochastic component that may account for diverse dysregulations associated with aging.

Age is determined almost certainly by the detailed pattern of methylation and other epigenetic markers, not simply the crude quantity of methylation. And yet there is evidence that senescing cells are characterized by progressive demethylation, so that chromosomes in younger cells tend to be more methylated than in older cells [46, 47]. The possibility that demethylation may be an aging clock was first proposed by Bowles [48] based on the fact that "aging is accompanied by DNA demethylation" [49-51]. In fact, the animal genome loses practically all 5-methylcytosines during its life, the rate of the loss being inversely proportional to maximal lifespan of the species [51]. The same occurs in cell cultures, again the rate being inversely proportional to the cell lifespan (Hayflick limit) [51-53].

Skulachev also notes a connection between oxidation, which has often been recognized as a stochastic marker for aging, and methylation. In his schema, oxidation is a more fundamental aging clock (rather than demethylation leading to oxidation, as I argue here). "Oxidation by ROS of the guanine DNA residues to 8-hydroxyguanine strongly inhibits methylation of adjacent cytosines [54]. Antioxidants, on the other hand, cause DNA hypermethylation [55]. According to Panning and Jaenisch [56], DNA hypomethylation activates *Xist* gene expression in the X chromosome, which correlates with a dramatic stimulation of apoptosis. All these observations may be summarized by the following chain of age-related events: ROS → DNA demethylation → apoptosis → aging" [53].

Drugs targeted to sirtuins [57] have found some earlier success in extending life span by indiscriminate silencing of gene expression. The mechanism of sirtuins is mediated via histone de-acetylation rather than methylation, but the ease with which simple silencing of genes could extend life span is suggestive. Also, protein-restricted and methionine-restricted diets retard aging [58], presumably by dialing down expression of many genes indiscriminately.

TESTING THE HYPOTHESIS. MEDICAL IMPLICATIONS

This hypothesis – that the body's age is stored within the cell nucleus as a methylation pattern – suggests a program of research, and an anti-aging strategy.

Interventions based on methylation will require both a detailed, automated reading of the methylation state of the genome, and a means of transcribing a youthful profile into chromosomes *in vitro*.

The former is already fairly well developed. Heyn et al. [47] report transcription of the methylome using micro-arrays. The latter may be far more challenging. Methyl transferases are able to methylate targeted genes, but details of the biochemistry that guides the transferases to their target are not yet understood [37].

More accessible might be interventions to increase methylation broadly. This is a life extension strategy that has been made to work in flies [41] but not yet in mammals. S-Adenosyl methionine (SAM) is the basic methyl donor of all eukaryotes. Simple supplementation with SAM has been found to relieve arthritis and depression symptoms [59], and SAM has been shown to protect methylation levels in radiation-challenged mice [60], but SAM has not been found to extend life span in rodents. Johnson [37] catalogs some nutrients that have been associated with reduced methylation, but none with enhanced methylation. They stress that we are yet at an early stage of knowledge concerning the relationship between methylation and aging, and it is not yet proven even that alterations of the methylation state are a cause and not simply a product of aging. Nevertheless, they propose methylation as a promising avenue for foundational and clinical research, and I concur.

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