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Studies that Shed New Light on Aging

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Abstract—I will first discuss how all aging models that assume that the aged cell has irreversibly lost its youthful capabilities through such mechanisms as accumulated dysfunction, accumulated damage, and/or accumulation of toxic byproducts of metabolism have been shown to be incorrect. I will then briefly discuss models of aging and propose an experiment that would distinguish between those models and provide a basis for organismic rejuvenation.

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By the turn of the 21st century, gerontologists and biologists reached a consensus "evolutionary theory of aging" [1-3], putting aging research into the mainstream of biological research. This so-called theory, simply put, states that because continued selective pressure tends to make the lifespan of a sexually-reproducing organism longer – as a longer-lived organism produces more offspring – it can be assumed that mechanisms retarding aging are insufficient to allow organisms the long lives evolutionary theory predicts. The theory assumes that an aged cell is irreversibly damaged as a result of life's slings and arrows, leading to the currently accepted paradigm of "wear and tear" as the cause of aging.

The "wear and tear" paradigm of aging maintains that as an organism ages, the incidence of cellular damage and toxic metabolic byproducts eventually exceeds the organism's ability to repair or remove them, leading to their accumulation [4]. This, in turn, results in the increase in the number of senescent cells and a decrease in effective stem cell populations, or an alteration of their potency [5]. So aging is characterized as the accumulation of damage leading to dysfunction. Metaphorically, the aging of an organism is like that of an automobile, eventually as parts wear out and malfunction more quickly than they can be repaired or replaced, the automobile becomes a jalopy destined for the junkyard. This is assumed to be the same case regarding cells, even stem cells, and consequently the tissues, organs, organ systems, and the organism whose functioning is based on those cells.

CELLULAR REJUVENATION IMPLIES STOCHASTIC THEORIES OF AGING CONFUSE CAUSE AND EFFECT

In spite of inconsistencies and improper assumptions, these current leading "evolutionary" theories of aging are widely accepted models yet, to call them "theories", is normally a scientific assessment of their proven truth, which is not the case [6]. As this paper will show, the hypothesis that aging at the cellular level is the product of the accumulation of irreparable damage and/or uneliminable toxic substances has been tested and rejected in numerous studies. Simply demonstrating that cells can be rejuvenated by exogenous factors is logically equivalent to demonstrating that cells are not irreparably damaged by aging, in direct contradiction to the assertion that aging is the result of irreparable damage. If cells are not irreparably damaged by aging, then what makes them age and accumulate damage? As we will show, it is becoming clear that the accumulation of damage is an effect of aging and not its cause. This confusion of cause and effect is evinced in many aging "theories" based the many phenomena correlated with aging. So mitochondrial dysfunction, ROS (reactive oxygen species) production, telomere shortening, and DNA damage accumulation – each has been separately regarded as "the cause" of aging [7], yet they are all more properly regarded as effects of aging: the demonstration that each of these "causes" of aging are reversed by cellular rejuvenation shows that those are all processes that are not its cause but are all

consequences of aging, the result of cellular decisions, but decisions that can be, as we shall see, reversed. All stochastic models that assume the hypothesis that aging results from the irreversible loss of information to entropy are shown to be incorrect by the demonstration of cellular rejuvenation: the hypothesis that information is irretrievably lost through aging must be rejected as the information for a total renewal was not lost in the aged or senescent cell: it was demonstrably recovered. As we will show, evidence points to aging being a programmed process controlled, in mammals, through factors present in their blood. The ultimate control of the process is epigenetic, but it is coordinated across the body by soluble factors and juxtacrine interactions. I believe the process is a continuation of the developmental program, with a beginning and an end.

Kirkwood's [8] statement "but, if programmed, the programming is very loose, because there is a large variation in the rates of senescence of individual cells within the population", is close to what we believe to be true, in that an organism's lifespan is governed by a very loose programming with many environmentally-influenced decision pathways that may extend or diminish lifespan, but inevitably (except for organisms showing negligible senescence, or replaying earlier developmental stages like *Turritopsis nutricula*) drives an individual from zygote through reproductive adulthood and to subsequent senescence and death. To suggest that aging changes are to a great degree preordained is to take a position that sounds more hopeless than the various variations of "wear and tear" (stochastic) theories which, at least, grant the possibility of interfering with damage production and/or accumulation. However, as we will show, several studies indicate that the exogenous factors can reset the age phenotype of body cells, tissues, organs, and possibly organisms, this view of aging leads to the possibility of recovering youth – that very "fountain of youth" that Dr. Kirkwood told us was to go the way of the perpetual motion machine – an impossibility [9].

AGE-RELATED TRANSCRIPTION PROFILES AND THE THEORIES OF AGING

The stochastic theories of aging suggest a general decline [2] in all aspects of the organism – it is suggested by all the wear and tear theories that the cell ultimately becomes dysfunctional to the stage where it cannot perform its duties nor even maintain itself and dies. It is expected under such a paradigm that the cell deteriorates with age, much as an automobile does. As opposed to this, an explanation more consistent with observed biology is an epigenetically controlled program that is an approximately life-long extension of the developmental program that begins with fertilization [7]. If that is the case, then aging may be evolutionarily selectable as nature may be selecting a successful program and not a collection of genes, or for that matter individual, "selfish" genes.

Evidence for this view comes from the technological innovation that allows the simultaneous analysis of the rates of transcription of tens of thousands of genes using DNA microarrays [10-12]. These sorts of studies show age-dependent changes in the regulation of thousands of genes. The study of Stuart Chambers [13] shows that there are marked age-dependent, order of magnitude differences in the transcriptional rates of many genes, and of these age-regulated genes, the regulation must be seen as anti-homeostatic. An example of this is the down-regulation of DNA repair genes in mice [13] at ages at which DNA defects show increased frequency and DNA repair becomes inefficient [14]. Other examples will be considered later. One would certainly suspect that down-regulating the transcription rates of repair enzymes at or just prior to the time of increased DNA damage accumulation and the documented decreased ability for DNA repair ("inefficient repair" [14]) must have a causal connection. Yet homeostasis is an active process that at the level of the cell tries to preserve integrity – turning down DNA repair activity at the time of an increase in DNA damage (by ROS, "leaky" mitochondria, etc.) is exactly contrary to the principle of homeostasis (even failing to up-regulate DNA repair when there is DNA damage is at least non-homeostatic) and demands an explanation. The explanation of the hypothesis called "antagonistic pleiotropy" would be that some pleiotropic protein that once acted towards the cell's benefit now operates (at its evolutionarily untouchable post-reproductive stage of deterioration) to its detriment – but what we observe is not a change in the character of genes, but a quantitative change in their appearance in the cell – and that change is opposite to what the principle of homeostasis demands! While Chambers attributes those negative changes, such as genetic dysregulation and the loss of genetic suppression to entire segments of chromosomes, to epigenetic dysregulation, the very specific timings of age-dependent changes in transcription rates of aging-related genes, apparently correlated with developmental stages in the post-adult lifespan, implies a programmatic decision to down-regulate cellular maintenance and repair systems aging damage rather than a stochastic unraveling of epigenetic control. The age-coordinated expression of specific genes appears to result in the very dysfunctions that their lack or excess might reasonably be expected to give rise to: decreased synthesis of DNA repair proteins might be expected to give rise to the accumulation of DNA defects, excesses of proinflammatory cytokines should lead to generalized inflammation, increased production of amyloidogenic proteins ought to produce amyloidoses (including Alzheimer's disease) – processes associated with aging [13].

It may be safely assumed that cellular deficiencies including the progressive loss of proliferative potential and potency in stem cell and progenitor cell populations

[15] and the appearance of "senescent cells" lead to the deficiencies seen at the higher levels of tissues, organs and organ systems and the organism itself [16, 17]. This occurs because, among other disabilities incurred by aging, stem cell populations lose the ability to replenish somatic tissues, some mitotic cells become senescent and actively produce noxious products; both paths ultimately culminating, at the organismic level, in the diseases of aging and death.

Seemingly, the increase or decrease in functionalities provided by age-related gene transcription are responsible and the age-related increase in the number of senescent cells are responsible also for the diseases of aging (e.g. cardiovascular disease, cancer, diabetes, dementia, and pneumonia) [18]. Which basically means that a cell deliberately diminishes its repair and maintenance capabilities, it deliberately increases its ROS production, it deliberately decreases the efficiency of its DNA repair, it deliberately decreases the sirtuins it requires for chromatin maintenance, it deliberately increases the noxious products it vents to the surrounding tissues, it deliberately increases the production of proinflammatory cytokines, matrix metalloproteases – or hard to clear amyloid forming proteins. How can any of this be accounted for by any theory that assumes homeostasis is a governing principle in biology – to what perceived threat or lack thereof does the cell turn down its protective mechanisms at the beginning of middle age [13] – what explanation can the "wear and tear" hypotheses give to the cell turning down repair systems as damage increases?

Before outlining a new approach to investigating aging, I will first put to rest those aging models that assume, for whatever reason, that the aged cell has irreversibly lost its youthful capabilities through stochastic mechanisms; that is, that the cell is irreparably damaged through aging. I will then briefly propose a new model of aging as a continuation of development, discuss its relationship to diseases of aging and, finally, propose a possible means by which cellular rejuvenation therapy might be tested. At this time when the proposed costs of medical services to be provided by health services for seniors is reaching astronomical levels, a way of delaying those costs would gain nations much needed time.

REJECTING THE "WEAR AND TEAR" HYPOTHESIS OF AGING

Let us now debunk this paradigm of "wear and tear" for biological aging, using experimental evidence.

If aging results from the accumulation of damage or of toxic metabolic byproducts, then chronologically old cells should be damaged beyond the cell's ability to repair itself. This is not the case, however based on clear results from decades of experimentation. Aging at the cellular, tissue, organ, and organismic levels have been reversed by exposing the tissues of old animals to a young environment [19].

If cells can be rejuvenated by intrinsic means as a result of extrinsic signaling, then the presumption that they are damaged beyond their ability to repair themselves is logically false. Thus, all theories of aging based on the "wear and tear" paradigm are proven wrong by experimentation. If a cell can be rejuvenated, it cannot be damaged beyond repair by aging.

Let us begin with the weaker evidence and move towards the stronger.

1. The nuclei of cells that have been passed in cell culture to the point at which they were senescent and incapable of further cell division have been used to clone normal animals. In this series of experiments, somatic cow fibroblasts were passed serially in culture until senescence and their nuclei were then extracted and placed into enucleated bovine ova. These ova were able to produce genetically normal offspring – this demonstrates that the nuclei of senescent cells are repairable at least. Apart from showing that there was no irreparable damage to these cell's nuclei, the experiment established that the aged nuclei proved fully capable of producing normal offspring (although the telomeres of cattle resulting from this were longer than normal) [20].

Similarly, induced pluripotent cells can be said to have been rejuvenated by the process used to produce them, by bringing them back to earlier developmental stages, it seems that we also reset their age phenotype. The studies of Lapasset et al. [21] showed that induced pluripotent cells derived from centenarians were rejuvenated such as to be indistinguishable from those derived from youthful cells using a set of criteria that include telomere length, mitochondrial function, gene expression profiles, mitochondrial efficiency, and ROS production. Those characteristics represent what we know of the age phenotype, so we must conclude that the age phenotype was set back as well as the state of differentiation. It should be noted here however that heterochronic parabiosis studies and cross-age organ transplant studies (to be discussed) show that the age phenotype can be affected independently of the state of differentiation, that is a cell can be rejuvenated without changing its state of differentiation [22]. The theory that increasingly dysfunctional, aging, mitochondria produce reactive oxygen and nitrogen species resulting in a death spiral of further dysfunction and destruction cannot be correct if mitochondria are "rejuvenated" by external signaling (showing that they are far from being irreparably impaired). That evidence together with all the other evidence indicates that cause and effect have been confused; mitochondrial dysfunction, ROS production, and telomere shortening are the effects of aging, not the causes. Age-specific transcription patterns are in accord with programmed aging, but not with stochastic aging, especially as the "programming" is exactly opposite to what would be expected from a homeostatic system.

2. Cross-age transplantation studies have shown that tissues and organs can be rejuvenated. The transplantation of tissues or organs of old donors into young recipients provides convincing evidence that old cells are fully capable of regaining youthful functioning when placed in the bodies of young animals [19]. Furthermore, it has been demonstrated that when an aged, involuted thymus gland is placed in a young body, it is rejuvenated and regains full functionality, even though it was originally in a senescent state [23]. If irreversible cellular damage is the cause of cellular aging, then restoring aged cells to youthful functioning by merely changing their environment would not be possible. So the aged cell is not irreversibly damaged.

3. Heterochronic parabiosis is a technique by which the circulatory systems of old and young animals are joined. As might be expected, based on the cross-age transplantation studies, the stem and progenitor cell populations are "rejuvenated" to the extent that they act more like younger stem cell populations. The rejuvenation taking place within the old animal was shown to be entirely dependent on factors present in the shared, hybrid blood supply [5]. Reciprocally, stem cell populations of the younger heterochronic partner have been shown to behave more like stem cells from older animals. More recent studies also show that a heterochronic parabiotic pairing between young and old rats resulted in increased neurogenesis and functional improvement of cognitive ability in the older parabiotic partner, making it appear closer to that of a younger animal, while the younger partner exhibits decreased neurogenesis and cognitive abilities, consistent with that of an older rat [24].

If aging at the cellular level were the result of the accumulation of damage and/or toxic byproducts of metabolism, cellular rejuvenation would not have been possible. If "wear and tear" were correct, rejuvenation by mere signaling would not be possible, but it is and therefore further adherence to such theories as postulate the irreversible degradation of a cell by stochastic processes is incorrect based on multiple lines of experimental evidence.

AGE PHENOTYPE OF STEM CELLS IS CONTROLLED BY SYSTEMIC SIGNALS

Though largely ignored by mainstream biology since the 1950's, the belief that lifespan is subject to evolutionary forces and may be programmed continues to be investigated [7]. Developments, both those contradicting the view that cellular accumulation of errors (stochastic damage) is the cause of senescence and those providing evidence supporting programmed aging have challenged the mainstream view. At this time, the evidence that aging is an actively programmed process appears to be compelling. And it is very much the same evidence that relegates "wear and tear" to the dustbin of history. The cross-age transplantation studies confirm that tissues adjust their cellular age phenotypes to be consistent with the age phenotype of their host. The heterochronic parabiosis studies indicating that both partners' (young and old) tissues tended to resemble each other in that the tissues of the older parabiotic partner appeared not quite as youthful as those of a fully young animal, nor the tissues of the younger partner as aged as those of the older partner. This hints at a concentration effect of these blood-borne factors.

An experiment to decide whether aging results from the increasing incompetence of aged cells or the environment of those cells might be done as follows: tissue from a young animal is transplanted into an old one, and tissue from an old animal is transplanted into a young one: if aging causes the increasing incapacity of cells, if old cells are damaged cells, we would expect the young tissue in old animals to fare well, while the old tissue placed into young animals – being defective, would not. However, the opposite is what actually occurred, transplants of young tissue into old organisms failed while old tissues transplanted into young animals produced successful grafts [17].

The observation that the stem cells from young animals behave as though they were senescent when transplanted into old animals confirms that it is the cells' environment rather than cell-intrinsic defects that result in the phenotype of an aging cell [25, 26]. If an organism's cellular age phenotypes were determined by extrinsic factors, the tissues of aging donors might respond to a young environment by becoming young. The answer was unequivocal in the case of muscle satellite cell transplants in rats, the old tissue in young rats did well, becoming like young muscle in terms of its ability to proliferate and repair muscle damage; and on the other hand, the young muscle transplanted into old rats showed both decreased proliferative ability and wound healing capacity – characteristics of "old" cells [25, 27]. Work with hematopoietic cells was more equivocal, but under proper conditions Harrison et al. [28] showed that hematopoietic stem cells were rejuvenated, totally capable of supplying the needs of the young mouse and regaining youthful regenerative capacity though they were derived from immune-deficient old mice. Careful controls ensured that organ regeneration was not the result of cells from the host but was entirely derived from the grafted tissues. At this point, two conclusions become clear: cellular age phenotype for some cell types at least is determined by systemic factors carried in blood, and cellular aging can and has been reversed, at least in some tissue types.

What happens in an organism's tissues does not necessarily happen to the entire organism. If aging was a result of systemic signaling and coordination amongst widely separated tissues, then surely the blood must transmit those signals [25]. If that is the case, then replac-

ing an older animal's blood, at least in part, by that of a younger animal's and vice versa should lead to a change in the age phenotype of the cells of both animals, such that the younger cells appear to age and the old cells seem "younger". These were the actual results obtained from experiments [5, 25]. If aging at the cellular level were reversed, would this lead to the rejuvenation of the animal at the level of the organism? And would it result in life extension? The unusual technique of "parabiosis" was used in the first experiments to investigate the possible rejuvenation of an animal. The technique consists of the surgical joining of the circulatory systems of two animals (and the animals themselves – shoulder to shoulder and hip to hip) so that they share a common blood pool. In 1957 McCay et al. [29] found that when a young rat was joined in such a manner to an old rat (heterochronic parabiosis) for a period of time, the old rat appeared younger. The evidence was equivocal however relying solely on the visual appearance of tissues (mostly cartilage) during autopsy. Some 15 years later, Ludwig et al. [30] used a more robust and quantitative experimental design to show that the older animals did, indeed, benefit from sharing the blood supply of the younger animal by demonstrating life extension. While this is certainly a confirmation of McCay's experiments, it could be argued, however, that during its parabiotic association, the young rat's body took over functions diminished by aging in the old rat, the young organs did double-duty in assisting or replacing the diminished functions of the old animal and so extended its life span. The experiments undertaken by Conboy et al. in 2005 [5] showed that stem cell tissues of the older rat were phenotypically younger than age-matched controls as measured by increased proliferation in response to wound-healing. The further experiments of Villeda et al. [24] showed that two organismic criteria of aging, neurogenesis and cognitive ability, were also reversed by parabiotic joining with younger animals.

BLOOD-BORNE FACTORS CONTROL THE CELLULAR AGE PHENOTYPE

Neither the cross-age transplantation studies nor the parabiosis experiments of Conboy et al. [5] yielded experimental evidence distinguishing whether positive factors (those promoting young phenotypes) or negative factors ("aging" factors) present in the blood of older animals or some combination of these positive and negative factors controlled cellular age phenotype. While it might have been youth-inducing factors provided by "young blood", which produced the observed rejuvenation of aged cells or a decrease in the concentration of negative, "aging-inducing factors" which caused the "rejuvenation" of old cells, this was not decidable through the experiments done. In their e-publication [31], Silva expresses their belief that aging is caused by the accumulation of negative factors. The studies of Zhangfa et al. [23] showed that the systemic environment of telomerase-negative mice became the major inhibitor of lymphopoiesis as their telomeres shortened; thus telomere shortening appeared to give rise to a systemic environment that caused apparent aging in terms of decreased lymphopoiesis.

In agreement with the effects of the systemic environment on aging, aged stem cells were rejuvenated by young plasma *in vitro*, and young stem cells were "aged" by *in vitro* exposure to plasma from old animals [32]. Stem cells derived from the young partners in heterochronic parabiosis lost proliferative potential compared to age-matched controls, while the cells of the older partner were physiologically younger than those of age-matched controls. Still, there is no conclusion about whether positive factors are increased in young blood, or negative factors decreased by dilution, or whether a combination of the two (presence of positive factors in "young" blood and the decreased concentration of negative factors found in "old" blood) resulted in rejuvenation. More recently, the exploration of the effects of parabiosis on the neurological aspects of aging implicated several cytokines that showed differentially increased concentration in the cerebrospinal fluid of old mice, and it revealed that at least one of these, CCL11 or "eotaxin", caused a decrease in neurogenesis and a decrease in cognitive ability when injected into the systemic circulation of young mice [24]. The works of Conboy, Carlson, and Villeda as well as all of the cross-age tissue and organ transplantation studies established that rejuvenation at the tissue and organ levels (the functioning of the brain) takes place when these tissues or organs are exposed to a younger systemic environment. There is now evidence that at least some compounds that are differentially elevated in the cerebrospinal fluid of aged animals (the "environment" of neural stem cells) can cause aging-like changes in an organism's age phenotype; at least one such chemical can cause aging-like changes when injected into the blood, identifying at least one substance differentially accumulating in aged animals that can be said to have a negative, aging-promoting effect [24]. It has been demonstrated using heterochronic parabiosis that all stem cell populations examined (using a small but important sampling) were rejuvenated by a young systemic environment. The well-controlled experiments of Conboy [5], Conboy and Carlson [32] in 2009, and later Villeda [24] (2011) established that factors carried in the blood are sufficient to produce changes in cellular age phenotype.

The experiments described above imply that the cell's age phenotype is determined not only by the presence (or absence) of factors in the blood, but also by the concentration of those factors. As shown in both the Conboy and Villeda heterochronic parabiotic experiments, the actual degree of rejuvenation of old tissue or the extent of aging of young tissue was intermediate

between full youthful phenotype expected in the young partner and the aged phenotype found in age-matched controls for the older partner. This may be because about half of the blood plasma is contributed by each partner. Furthermore, the thymus transplantation study of Zangfa et al. [23] showed that senescent, involuted thymuses became fully functional in the young animals into which they were transplanted. In this case, the transplanted organ was exposed to a fully youthful environment and assumed a youthful phenotype. It is unfortunate these studies did not measure changes, if any, in telomere length, mitochondrial functioning, gene expression patterns (although the transcription rates of some genes analyzed showed a youthful pattern), or in the number of senescent cells present, aging markers of interest, in the cells of rejuvenated tissues or organs. It should also be noted that in the case of cross-age transplantation the old organ (which had only a small fraction of the mass of the young donor into which it was transplanted) was rejuvenated rather than the young recipient prematurely aging – implying that the relative differences in mass determined the age state of the cells of such an asymmetric joining.

One possibility is that rejuvenation requires the presence (or absence or a required concentration) of a number of different factors; eotaxin was not likely the only contributing factor to the aging of the brain and loss of cognitive functioning in aged animals. Of the cytokines found to increase in concentration in the cerebrospinal fluid of aging animals, others have been shown to have aging effects [33]. Evidence that the reduced levels of at least one cytokine, IL-15, cause the symptoms of aging (sarcopenia, immune senescence, and obesity) [34].

It seems that one conclusion that is borne out by these studies is that if a cell is placed in a "young environment" or an "old environment" that cell will assume the age phenotype appropriate to its environment. And not to be coy, evidence indicates that an aged stem cell placed in a young environment will rejuvenate if given sufficient time.

Though the possibility of rejuvenation does not fit into the present aging paradigm, it has been demonstrated numerous times. The actuality of rejuvenation at the cellular level represents proof that the present aging paradigm is incorrect. That rejuvenation can occur at the level of the cell, tissue, and organ has been conclusively demonstrated, so that the lack of interest in exploring this phenomenon is surprising as it represents a potential therapy for the diseases of aging. It seems indisputable that aged tissues and organs become rejuvenated if bathed in the circulating blood of young animals for sufficient time.

So, the demonstrated ability of cells to rejuvenate and the fact that the circulating blood of young animals is sufficient to bring about rejuvenation of cells, tissues, and organs must make us reconsider any models of aging that do not allow for this phenomenon. Two possible models that would account for the observed aging phenomena are: 1) the accumulation, through aging, in the systemic environment of factors that produce the aging phenotype in cells and/or the progressive loss of factors that promote cellular "youthfulness", or a combination of the two; and 2) a blood-borne messaging system designed to ensure that the age phenotype of cells is appropriate to the age of the organism, though these two mechanisms may be the same.

In the case of the accumulation of deleterious factors or the loss of youth-promoting factors in the blood, one would have to posit a mechanism for why accumulation/reduction occurred. The second model makes an assumption that can account for both rejuvenation and aging; it posits that aging is a programmed process and coordination of cellular age phenotype throughout the body is achieved through the bloodstream. Whichever is the case, the experiments discussed give a clear indication that aged cells, tissues, and organs can be rejuvenated, and how this can be accomplished.

POTENTIAL FOR ORGANISMIC REJUVENATION

It has been determined that if a stem cell (at least) is in an "environment" of a certain age ("young" or "old"), it will assume the age phenotype of the cells of its type at the age of the environment, given sufficient time. The questions remaining are: What is meant by "age phenotype"? What is meant by "environment"? And, how much time is "sufficient"?

A cell's "age phenotype" is taken to mean a set of characteristics that distinguish older cells from younger ones. It has been defined by various assays for increased dysfunction and decreased (mostly) proliferation as well as for various "markers" of aging as patterns of gene expression and epigenetic "marks" – but only clearly distinguishes old cells from young ones. Are there several age phenotypes or is there a continuous increase of age-related dysfunction? The work of Chambers et al. [13] showed that certain age-related genes were up- or down-regulated at particular stages of the mouse life cycle, corresponding to what we would call young adulthood, early middle age, late middle age, and old age; do the same age-specific distinctions exist in other characteristics associated with cellular aging such as telomere length, mitochondrial metabolism, redox environments, and proportion of senescent cells? Are there distinct age phenotypes apart from "young", "old", and senescent? An important practical question that needs to be answered directly is what happens to senescent cells in a young environment. The answer to these questions will have a profound impact on our understanding of aging – particularly on whether or not aging is a programmed process and more importantly, whether or not cellular age phenotype can be manipulated *in situ*, in an aged body so as to rejuvenate it.

That the environment found capable of rejuvenating cells *in vivo* was the blood or plasma of young animals is supported both by cross-age transplantation studies and by parabiosis experiments. Parallel *in vitro* work performed in the original Conboy experiments [5] showed that plasma obtained from the blood of young animals was sufficient to rejuvenate old stem cells in cell culture, and that young cells treated with plasma from old animals experienced accelerated aging. Furthermore, it was noted that young cells placed in an "old" environment appeared to age immediately [32].

Whether it is a question of removing deleterious substances from the blood plasma and stem cell niches of aged animals, or providing youth-promoting factors, or providing an internal environment of a certain "age", then the answer to organismic rejuvenation would be to exchange as much blood or plasma as possible to reduce the fraction of the original blood or plasma remaining. It should be noted that the "environment" in the case of heterochronic parabiosis consists of a mixture of the blood of both young and old parabionts so that both partners share a circulation that is about an equal mixture of young and old blood, while in cross-age transplantation, when the tissues or organs of an old animal are transplanted into a young body (or vice versa) – the recipient organ experiences blood that is entirely young or old. The near instantaneous change in a young cell placed in an environment of old plasma versus the much less profound changes in the cells of an organism that is the younger heterochronic parabiotic partner may result from the differences in concentration of factors found in the aged plasma it is exposed to. Using plasma exchange to replace parabiosis, it would appear that the greater the reduction of the original aged plasma (exchanging 87% of plasma requires two volumes of donor plasma), can be obtained, and this might have a faster and more complete rejuvenating affect.

At a recent Society for Neuroscience conference in New Orleans, Saul Villeda related an experiment wherein the mere injection of young plasma into old mice (at about 5% of total blood volume of plasma injected into a mouse for each injection, 18 such injections for a month) elicited significantly increased neurogenesis and cognitive functioning – this experiment (not yet published) indicates that positive, "youth-promoting" factors are also present in young serum as dilution of "aging factors" would not have taken place considering the small quantities of blood given. Indeed the results of the Villeda's experiments demonstrate that factors present in old plasma both negatively and positively affect neurogenesis, synaptic plasticity, and cognitive functioning – should recommend plasma replacement for the treatment of age-related dementias. If it is the presence of factors accumulated in an old environment and/or the absence of factors (or changes in their concentrations), present in a young environment, plasma from young animals should be sufficient to rejuvenate all stem cell types that are rejuvenated by parabiosis or cross-age transplantation. The same holds true if the systemic circulation determines the age phenotype through a process of signaling. In that case, the blood or plasma of a young animal donor should contain all signaling factors needed to determine the age phenotype of the recipient's cells, at least initially, depending upon the biological half-life of those factors. The evidence that heterochronic parabiosis extends the upper limit of the species lifespan of the older parabiont, taken together with the evidence that the tissues of the older parabiont are rejuvenated, leads to the conclusion that significant, if not total, rejuvenation of the organism may take place. Hence, the entire organism might be rejuvenated by substituting the blood/plasma of a younger animal for its own. If the cellular age phenotype is determined by stochastic factors, there should be a single age phenotype, that of the young cell, but with varying degrees of impairment. However, if the aging process is programmed, and age phenotype is determined/coordinated by plasma-borne factors, then there will be a continuum of age states marked by particular patterns of gene transcription and of epigenetic marks. If such a situation obtains, than bathing cells in the plasma of a particular age will cause those cells to produce the transcription patterns, epigenetic marks, and mitochondrial ROS production that are specific to that age [10-12]. Such a programmed theory might assume that there are many cellular-age phenotypes, with transcriptional patterns providing age markers such that cellular age phenotypes might be broadly classifiable as markers of developmental stages (gastrula, toddler, early middle age, late middle age, etc.). The problem with this model, therapeutically at least, is that some or many of the important signaling molecules associated with age phenotype determination may have short half-lives, and they may be replaced with molecules made by the recipient's body that would signal cells to assume an older age phenotype. This situation can be solved by multiple or continuous plasma exchange for the time required for the recipient's cells to change their age phenotypes. It would seem that both the relative proportions of old vs. young plasma as well as the length of time of exposure are both variables in determining the cell's age phenotype. The demonstration by Villeda of the anti-aging effects of young serum tends to bolster the notion that whichever factors produce the youthful transformations of old animals remains in the plasma for sufficient time to have an effect.

HETEROCHRONIC PLASMA EXCHANGE (HPE) MAY BE AN EFFECTIVE SUBSTITUTE FOR PARABIOSIS

While it would appear that a series of exchange transfusions might be sufficient to rejuvenate a patient, the risks (http://www.nhlbi.nih.gov/health/health-topics/

topics/bt/risks.html) and costs [35] associated with complete whole blood transfusions make it unacceptable for therapeutic intervention on the massive scale that would be needed. However, the demonstration, *in vitro*, of the ability of young plasma to rejuvenate old stem cells and even the brain, leads to the possibility that the *in vivo* exchange of the plasma of an old animal for that of a younger animal might be sufficient to bring about rejuvenation if provided with the proper schedules of exchanges. Like the blood of young organisms, "young" plasma would lack those substances that induce aging and contain those that permit or encourage cellular youthfulness, assuming that no part of those factors is carried in the cellular portion of blood. Experiments, using both whole blood or plasma replacement, or a schedule of such replacements (see below) between young and old individuals of mouse or rat strains could test whether blood or plasma exchanges will lead to rejuvenation. Much of the costs and risks of blood exchange transfusions would be eliminated by using plasma exchange.

Plasma exchange is a form of plasmapheresis wherein blood is removed from the body, separated by either centrifugation or filtration into a cellular fraction and plasma – with the cellular portion is either mixed with donor plasma or a plasma substitute and returned to the patient; the patient's plasma is discarded. Plasma exchange is used to treat many conditions that benefit from the removal of plasma or substances from plasma including autoimmune antibodies and toxins [36].

It now seems clear that organismic aging is controlled by genetic and epigenetic pathways and the exploration of these pathways is being pursued by a number of laboratories. It is also clear that various cytokines and cellular proteins are involved in the signaling pathways that cause aging in the same way that they drive development at all stages of human life. These include IGF-1, Notch, and WNT signaling pathways, which have all been implicated as being in pathways that appear to coordinate aging across tissues and organs. The IGF-1 pathway, in particular, appears to cause the aging of bone marrow cells, while it retards the aging of muscle satellite cells; hence, whatever the signaling hierarchy is, IGF-1 is not on top. To establish what factors and at what concentrations are required to reverse or induce aging might take decades, **but we do not need further elucidation of this phenomenon in order to apply it**. It appears that the plasma of young animals is sufficient to cause cellular rejuvenation of old stem cell populations. An analogy with electricity is in order: we used electricity to light our homes and drive our machines well before we had any idea of what electricity was; we knew what its effects were and used those effects to our own advantage. That said, as we know that the blood plasma of young animals can rejuvenate the cells of old mammals, it should be the case that the plasma of young humans will also rejuvenate human cells. Even if not all stem and progenitor cell populations are rejuvenated, rejuvenation of those stem cell populations that have already been shown to be capable of being rejuvenated (e.g. muscle satellite cells, liver progenitor cells, bone stromal cells, and hematopoietic stem cells) would be sufficient to markedly increase the quality of life of old people and likely result in an increase in the duration of youthful health. This "rejuvenation therapy" would include the near complete replacement of an older person's plasma with that of a young person (i.e. heterochronic plasma exchange or HPE) using a schedule sufficient to allow "cleansing" of stem cell niches in order to rejuvenate stem cell populations. Plasma exchange is an approved medical procedure; the single, simple variation of exchanging old blood plasma for young, could be readily accommodated by existing protocols and the procedure could be tested on people tomorrow. Prudence cautions us to try animal experiments first to test the principles and the extent of rejuvenation as well as its safety, yet the technology of plasma exchange has already been shown to be safe and the age of the plasma donor has never been material. Also, while the effectiveness of plasma exchange in "rejuvenating" cells has yet to be demonstrated in humans (though it should logically follow from experiments performed to date), the entrance into senescence of a significant portion of the populations of all industrialized nations at this time tells us to hurry and demonstrate HPE in animals and assess it on human volunteers as soon as possible.

FURTHER EXPERIMENTAL WORK

Establishing that blood exchange can functionally replace heterochronic parabiosis would be the first step. (If it could, that alone would allow researchers to examine the question of environmental effects on cellular age phenotype in a much easier and more controllable environment than by parabiosis.) What follows that would depend on whether or not plasma exchange substituted for transfusion exchange. If it did not, there would be the requirement of blood fractionation and "add-back" experiments to determine which cellular components of blood were required. Assuming that either transfusion exchange or plasma exchange can replace parabiosis, the next step would be to develop a time-course of changes in cellular age phenotype following either blood or plasma exchange in order to develop a schedule of exchanges necessary to produce "rejuvenation". As cellular aging and senescence appear to be behind all diseases of aging – the object of a "rejuvenation therapy" resulting from plasma or transfusion exchanges could be any or all of the diseases of aging.

It will be important is to establish the time course of blood/plasma-induced cellular changes in order to determine how long young blood/plasma needs to be in contact with these cells in order to affect a change in their age phenotypes. The heterochronic parabiosis studies of

Conboy et al. [5], Carlson et al. [27], and Villeda et al. (2011) [24] all seem to point to an intermediate level of rejuvenation when organisms are exposed to the combined blood of young and older parabionts. We find that the rate of proliferation of the stem cells of rejuvenated organs and tissues is somewhat less than that of chronologically young animals, though more than those of age-matched controls. It may be that stem cells "rejuvenated" by this procedure achieve an age phenotype that is somewhat between young and old. This should be investigated in cell culture comparing blood/plasma derived from animals of an intermediate age to mixed plasmas (young/old); if aging results from a simple change in concentration of positive or negative factors, then appropriate mixing of old and young plasmas should result in cellular rejuvenation equivalent to that achieved using the plasma of an animal of intermediate age. In addition, it will be most interesting to see whether stem cells from old or young donors reach the same state when raised in plasma obtained from animals of a particular age – whether, indeed, the cell's history or environment contributes the greater share to aging.

It would also be useful to see which cell types, stem and progenitor cells, as well as other cycling cell types are rejuvenated. Experiments with plasma fractionation will begin to tell us about those factors that "cause" aging/rejuvenation and these experiments can be performed *in vitro*.

Moreover, it would be interesting to note the effects of "young" plasma (plasma derived from young animals) on senescent cells. Cross-age transplantation studies show that old tissue is rejuvenated in a young host, but they give no information on what happens to the senescent cells, which are invariably part of the tissues of an aged organism. Are senescent cells rejuvenated or eliminated or do they remain as they were? And if eliminated, is the elimination accomplished by the young immune system or is it intrinsic, as in death by apoptosis? And of course plasma fractionation experiments could determine what the age-changing factors are.

While the parabiosis experiments are very difficult, *in vitro* experiments using stem cells and plasma derived from animals of particular ages should not be.

It has been demonstrated that aged cells can be rejuvenated, so the lack of attention that the aging research community has devoted to this most important phenomenon is surprising and must be remedied before millions more die of age-related diseases.

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