Review article

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The Fountain of Youth: A tale of parabiosis, stem cells, and rejuvenation

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Abstract: Transfusion (or drinking) of blood or of its components has been thought as a rejuvenation method since ancient times. Parabiosis, the procedure of joining two animals so that they share each others blood circulation, has revitalized the concept of blood as a putative drug. Since 2005, a number of papers have reported the anti-ageing effect of heterochronic parabiosis, which is joining an aged mouse to a young partner. The hallmark of aging is the decline of regenerative properties in most tissues, partially attributed to impaired function of stem and progenitor cells. In the parabiosis experiments, it was elegantly shown that factors derived from the young systemic environment are able to activate molecular signaling pathways in hepatic, muscle or neural stem cells of the old parabiont leading to increased tissue regeneration. Eventually, further studies have brought to identify some soluble factors in part responsible for these rejuvenating effects, including the chemokine CCL11, the growth differentiation factor 11, a member of the TGF-β superfamily, and oxytocin. The question about giving whole blood or specific factors in helping rejuvenation is open, as well as the mechanisms of action of these factors, deserving further studies to be translated into the life of (old) human beings.

Keywords: Blood; Brain; CCL11; GDF11; Liver; Muscle; Oxytocin; Parabiosis; Rejuvenation

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1 Introduction

In the 16th century, the famous Spanish explorer and conquistador Juan Ponce de León led the expedition around the Caribbean islands and eventually into Florida to find the Fountain of Youth, a magical water source supposedly capable of reversing the aging process and curing sickness [1]. Although the explorer made no mention of the Fountain of Youth in his letters, led by the rumors, the expedition continued the search and many perished. The Fountain was nowhere to be found, as locals were unaware of its exact location. Fontaneda wrote in his memories: "So earnestly did they engage in the pursuit, that there remained not a river nor a brook in all Florida, not even lakes and ponds, in which they did not bathe; and to this day they persist in seeking that water, and never are satisfied. [...] ...and it ended in all that numerous people who went over to Carlos forming a settlement: but to this day youth and age find alike that they are mocked, and many have destroyed themselves".

This is just one example of the continuous search human beings have dedicated to finding a way to live forever. Interestingly, another commonly cited approach for rejuvenation was attempting to transfer the warmth and fluids of youth from young people to old. Some examples of this approach were sleeping with virgins, a practice prescribed also by scientific physicians during the 17th and 18th centuries [2], or bathing in or drinking blood [3].

Blood, plasma, and their derivatives are what modern medicine has produced to aid stem cell function and tissue regeneration and repair. Platelet-rich blood derivatives, such as platelet-rich plasma (PRP) and platelet-rich fibrin, produce and deliver growth factors with antiapoptotic and angiogenic properties, augmenting the regenerative capacity of stem and progenitor cells, either resident locally or administered exogenously [4]. PRP has become popular for use in various orthopedic surgical procedures to treat different conditions including osteoarthritis [5, 6], in plastic surgery to improve graft survival [7, 8] and to treat impending skin necrosis [9]. Thus, no doubt that the blood and derivatives can be employed with success in

strategies of regenerative medicine, but even for the 'holy grail' of rejuvenation - the reversal of the aging process.

2 A tale of parabiosis

The claim that blood can rejuvenate our organs has been revitalized by one research group at the Stanford University School of Medicine in 2005 [10] and 2010 [11]. These studies stemmed out from observations which show that tissue regenerative capacity declines with age. In tissues such as muscle, blood, liver, and brain this decline has been attributed to a diminished responsiveness of tissue-specific stem and progenitor cells [12-15]. However, aged muscle successfully regenerates when grafted into muscle in a young host, but young muscle displays impaired regeneration when grafted into an aged host [16, 17]. Either local or systemic factors could be responsible for these reciprocal effects. In order to test whether systemic factors can support the regeneration of tissues in young animals and/or inhibit regeneration in old animals, the the paper by Conboy and colleagues of 2005 reported an experimental setup in which – in contrast to transplantation – regenerating tissues in aged animals are exposed only to circulating factors of young animals, and vice versa [10]. Thus, they established parabiotic pairings between young and old mice (heterochronic parabioses), with parabiotic pairings between two young mice or two old mice (isochronic parabioses) serving as controls (Fig. 1). In parabiosis, two mice are surgically joined, such that they develop a shared blood circulation with rapid and continuous exchange of cells and soluble factors at physiological levels through their common circulatory system [18]. Parabiosis was invented in 1864 by the physiologist Paul Bert in order to see whether a shared circulatory system was created. Clive McCay, a biochemist and gerontologist at Cornell University in Ithaca, New York, was the first to apply parabiosis to the study of ageing, but this technique fell out of favour after the 1970s, likely because many rats died from a mysterious condition termed parabiotic disease, which occurs approximately one to two weeks after partners are joined, and may be a form of tissue rejection. Only at the beginning of the XXI century, Irving Weissman and Thomas A. Rando at the Stanford University brought parabiosis back to life, to study the movement and fate of blood stem cells.

The Stanford group investigated muscle regeneration and liver cell proliferation in the parabiosis setting. After muscle injury, muscle regeneration was studied by the formation of myotubes expressing embryonic myosin heavy chain, a specific marker of regenerating myotubes in adult animals. Five days after injury, muscles in young animals in both isochronic and heterochronic parabioses had regenerated robustly. In contrast, injured muscle from old isochronic parabionts regenerated poorly. Notably, parabiosis with young mice significantly enhanced the regeneration of muscle in old partners. The regeneration of aged muscle was almost exclusively due to the activation of resident, aged progenitor cells, and not to the engraftment of circulating progenitor cells from young partners (as judged by the presence of less than 0.1% of green fluorescent protein [GFP] expressing cells derived from young partners transgenic for GFP). Since the loss of muscle regeneration with age is in part due to an age-related impairment in the up regulation of Notch ligand Delta after muscle injury [12], Delta expression was also studied. Notably, satellite cells from the aged partners of heterochronic parabionts showed a marked up regulation of Delta, comparable to that found in their young partners and in young mice not subjected to parabiotic pairings (Fig. 2).

In the case of liver studies, and as in muscle, while proliferation of albumin-positive cells in old isochonic parabionts was less than that observed in young isochronic parabionts, parabiosis to a young partner significantly increased hepatocyte proliferation in aged mice. As also in muscle, the enhancement of hepatocyte proliferation in aged mice was due to resident cells and not the engraftment of circulating cells from young partners. The

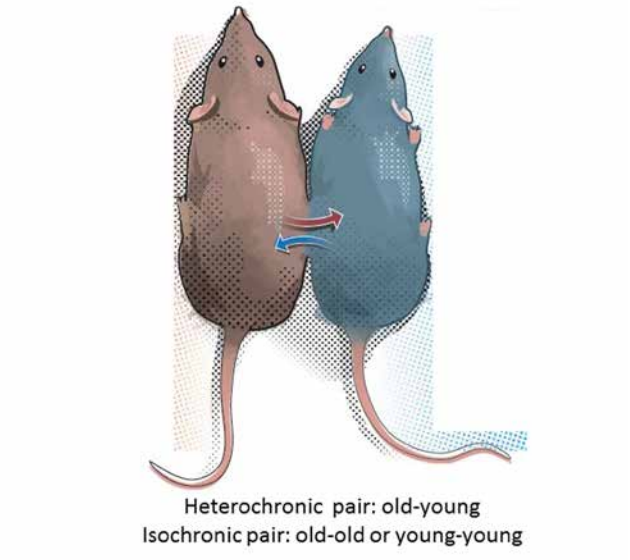

Figure 1: Parabiosis. Two mice are stitched together sharing a common bloodstream. Heterochronic parabiosis is when a young mouse is surgically joined to aged partners, while isochronic parabiosis is referred to pairs of young-young or old-old animals. Modified from ref. [36] with permission of Nature Publishing Group.

decline of hepatocyte progenitor cell proliferation is due to the formation of a complex involving cEBP-α and the chromatin remodeling factor brahma (Brm) that inhibits E2F-driven gene expression [19]. In parallel with the effect on hepatocyte regeneration, the formation of cEBP-α-Brm complex was detected in livers from old heterochronic parabionts but not from young isochronic ones, and the complex was diminished in old heterochronicparabionts (Fig. 2). Finally, in muscle and liver, they noticed a reduction of progenitor cell proliferation in young mice after parabiotic pairing with old mice, suggesting that old mice are enriched with inhibitory factors which are diluted upon parabiosis. Overall, this data indicated that there are systemic factors that can modulate the molecular signaling pathways critical to the activation or inhibition of tissue-specific progenitor cells, and that the systemic environment of a young animal is one that promotes successful regeneration, whereas that of an older animal either fails to promote or actively inhibits successful tissue regeneration. Finally, this work also demonstrated that tissue-specific stem/progenitor cells retain much of their intrinsic proliferative potential even when old, but that age-related changes in the systemic environment and/or niche in which progenitor cells reside, preclude full activation of these cells for productive tissue regeneration.

In the paper of 2010, Wagers and colleagues tried to figure out what is the role of local micro environmental niche-related and systemic factors in ageing of hematopoietic stem and progenitor cells (HSPCs), using the in vivo parabiotic mouse system and studying HSC frequency and number of long-term HSCs (LT-HSCs). Congenic markers were used to distinguish HSCs from aged versus young partners. Ageing is accompanied at the level of bone marrow by a considerable expansion of HSPCs coupled paradoxically with a reduced capacity for blood reconstitution and skewed differentiation potential after transplant [20-23]. Aged-heterochronic parabionts showed significant reduction of LT-HSCs, which approached normal 'youthful' levels. Notably, this effect arose from changes in the aged HSC population itself and not to trafficking of 'young' cells to the aged partners' marrow. Moreover, heterochronic parabiosis also induced recovery of LT-HSC function in aged mice, as evidenced by engraftment potential and restoration of youthful ratios of B lymphoid to myeloid cells. As with HSCs, both the frequency and total number of osteoblastic niche cells isolatable from aged mice were increased compared to young controls. In vitro experiments of interaction between young bone marrow cells with aged osteoblastic niche cells also showing expansion of HSCs, suggested that the HSC rejuvenating effects of heterochronic parabiosis occur indirectly – by reverting age-related changes in osteoblastic niche cells. Indeed, osteoblast frequency and number were restored to youthful levels when aged animals experienced in heterochonic parabiosis a young systemic environment. Moreover, niche cells isolated from aged-heterochronic parabiosis showed a significantly reduced capacity to cause HSPC accumulation, in contrast to niche cells from aged-isochronic parabiosis. Interestingly, osteoblast niche cells isolated from young heterochronic parabionts induced a slight expansion of young HSPCs as compared to the ones from young-isochronic parabionts. These in vitro studies suggested a reciprocal effect of the aged circulatory environment on niche activity in young heterochronic partners and indicate that systemic signals restore aged niches. Further experiments have been done, aimed at evaluating the ability of young HSPCs to reconstitute hematopoiesis. The results, demonstrated that, similar to the impaired engraftment function of naturally aged HSCs [20], young HSPCs exposed in vitro to aged-isochronic niched cells

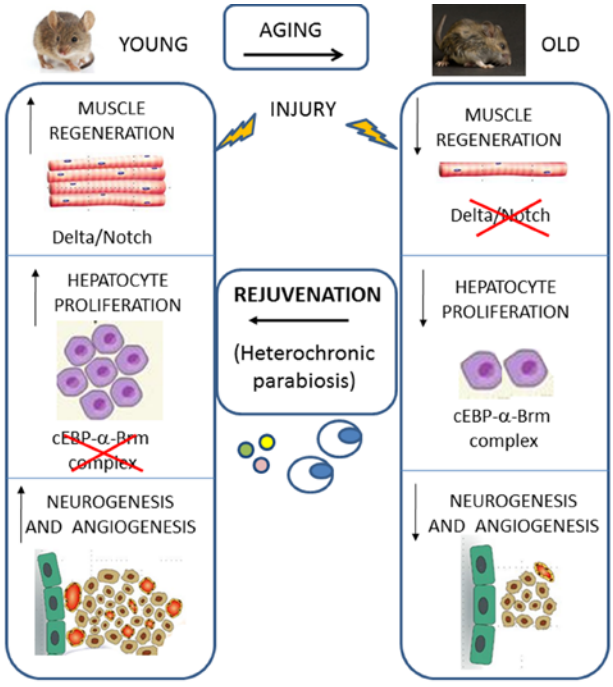

Figure 2: Ageing of muscles, liver and brain in old mice and rejuvenation by heterochronic parabiosis. The regeneration of skeletal muscle upon injury is linked with the up regulation of the Notch ligand Delta, that is lost with age (upper panels). Hepatocyte proliferation in young animals correlates with the decrease of cEBP-α-brahma (cEBP-α-Brm) complex as compared with aged mice (middle panels). While young animals can increase their neurogenesis and angiogenesis in the subventricular zone of the brain, where neural stem cells are present, aged animals cannot (lower panels). In principle, the heterochonic parabiosis reverts all phenotypic and molecular hallmarks of ageing by transferring soluble factors and cells.

exhibited a reduced capacity for hematopoietic reconstitution. This indicates that interaction with aged osteoblastic niche cells is sufficient to induce defects in HSC function. However, aged heterochronic niche cells did not alter the reconstituting activity of young HSCs. Together, this data demonstrated that age-induced, functional alterations in HSC-regulatory niche cells can be reversed by young circulating factors. Overall, these findings further suggest that the rejuvenating effects of a young circulation on HSC are communicated indirectly – by signaling from rejuvenated osteoblastic niche cells.

In order to understand which factor is involved in the regulation of niche cell function, the authors sought to investigate whether insulin-like growth factor-1 (IGF-1) could play a role. IGF-1 has been shown to be an evolutionarily conserved ageing and longevity regulator [24]. In vitro and in vivo experiments demonstrated that local, not systemic, IGF-1 seems to induce ageing of HSC-regulatory niche cells, and that neutralization of IGF-1 signaling in the bone marrow microenvironment reverts age-related changes in osteoblastic niche cells that impair their appropriate regulation of HSCs.

Overall, these findings suggested that while under youthful conditions osteoblastic niche cells promote homeostatic stem-cell maintenance, they are altered by ageing such that instead allow the enhanced accumulation of dysfunctional HSCs. These age-specific alterations in niche cells seem to be signaled by an uncharacterized circulating factors that act in part by altering IGF-1 signaling in the niche cell themselves (Fig. 3). It is likely that IGF-1 has not a major role for all the aged tissue, since while its role in the osteoblastic niche is age promoting, in contrast in skeletal muscle local expression of IGF-1 maintains regenerative capacity in aged animals.

In October 2010, three of the four authors, including Amy J. Wagers, retracted this paper, in particular for the role of the osteoblastic niche cells in the rejuvenation of HSCs in aged mice [25]. It was found that the first author manipulated the images for bone nodules formed in osteoblastic niche cells from young and aged mice (Retraction Watch, http://retractionwatch.com/2012/08/29/ori-finds-harvard-stem-cell-lab-post-doc-mayack-manipulated-images/). Thus, further confirmation about his issue should be obtained, considering also that the parabiosis model was exploited for studying rejuvenation of other old organs. Indeed, two papers subsequently appeared showing that exposing ayoung mouse to an old systemic environment can inhibit myogenesis [26] and neurogenesis [27].

In 2013, the team led by Amy J. Wagers, published another work by which they demonstrated using the parabiosis model, that age-related cardiac hypertrophy can be reversed by exposure to a young circulatory environment with only 4 weeks of parabiosis [28]. The measurement of blood pressures and of circulating levels of angiotensin II and aldosterone in the various groups, clearly demonstrated that the reversal of cardiac hypertrophy in old mice exposed to a young circulation could not be explained by a simple reduction in blood pressure or in the modulation of known effectors of blood pressure in the older mice. Interestingly, heterochronic parabiosis induced no changes in heart weight-to-tibia ratio, cardiomyocyte size, or blood pressure in young mice joined to aged partners. This data implicated an antihypertrophic factor produced by young mice (rather than dilution of a prohypertrophy factor produced by old mice) in the cardiac remodeling induced by heterochronic parabiosis. In "sham parabiosis", whereby mice are surgically joined while leaving the skin intact, such that they do not develop a shared circulation, no significant differencein heart weight-to-tibia length ratio in aged mice was found, further indicating that cross-circulation and exchangeof blood-borne factors are required for reversal of age-related cardiac hypertrophy. In order to identify these factors, a broad-scale proteomics analysis using aptamer-based technology revealed 13 analytes that reliably distinguished young mice from old mice. One of these candidates, the growth differentiation factor 11 (GDF11), a member of the activin/TGF-β superfamily, was confirmed in further analyses. GDF11 was reduced in the plasma of old isochronic compared to young isochronic mice and was restored to youthful levels in old mice after exposureto a young circulation. A daily 30-day treatment of old mice with GDF11 led to a significant reduction in the heart weight-to-tibia length ratio compared to the saline-injected control group. This data suggested that at least one pathologic component of age-related diastolic heart failure is hormonal in nature. However, the observed regression of cardiac hypertrophy in old mice exposed to a young circulation is unlikely to be attributable entirely to the replenishment of a single factor, and other factors should be recognized.

Two subsequent studies by Wagers and colleagues found that GDF11 boosted the growth of new blood vessels and neurons in the brain [29] and spurred stem cells to regenerate skeletal muscle at the sites of injuries [30]. In one of these papers [29], the mouse heterochronic parabiosis model revealed an increase in cerebral blood vessel volume and blood flow in response to young systemic factors, together with a higher self-renewal and differentiation in subventricular zone neural stem cell population, bringing an improvement in olfactory discrimina-

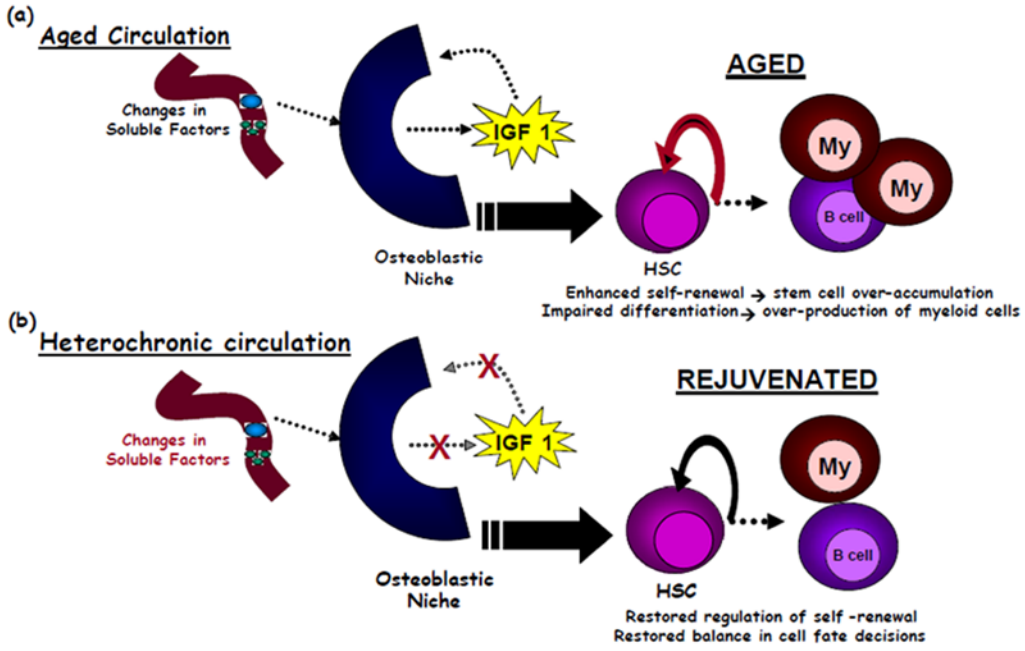

Figure 3: Proposed model describing age-related changes in osteoblastic cell niche and HSCs, and how these changes may be reverted by heterochronic parabiosis. Age-specific changes in autocrine or paracrine effects of IGF-1 on osteoblastic niche cells are signaled by circulating soluble factors which themselves change with age. IGF-1 signaling in aged osteoblastic niche cells (**a**) directly contributes to age-related dysfunction in HSCs, including HSC over-accumulation and skewed B lymphoid (B cell)/myeloid (My) fate choice. Following heterochronic parabiosis, or after neutralization of IGF-1 signaling in vivo (**b**), the "youthful" activity of aged niche cells is restored, such that they no longer induce over-accumulation or lineage skewing of HSCs. From ref. [11] with permission of Nature Publishing Group.

tion (Fig. 2). Furthermore, they found that GDF11 could increase blood vessel volume as well as neurogenesis in old mice. Interestingly, blood from 15-month-old mice did not decrease neural stem-cell populations in the young brain, whereas older blood (21 months) provoked a detrimental effect, suggesting that older the animals higher the accumulation of deleterious systemic factors and/or lower protective young factors. On the other hand, in the paper authored by Rando and Tony Wyss-Corey as senior scientists, the chemokine CCL11/eotaxin was identified as an age-related blood factor associated with decreased neurogenesis and impaired learning and memory in mice [27]. What is to be determined is if CCL11 interacts directly with neural progenitor cells during aging influencing their differentiation capacity, or it has indirect actions by interactions with other neurogenic niche cell types.

In the other paper by Wagers and colleagues [30], it was demonstrated that satellite cells sorted from aged-heterochronic mice had improved myogenic differentiation capacity as well as lower DNA damage when compared with satellite cells from aged-isochronic controls. As for the reversion of age-related cardiac hypertrophy [28], the treatment of aged mice with daily intraperitoneal injections of recombinant GDF11 for 4 weeks increased numbers of satellite cells with intact DNA, as compared with cells from aged mice receiving vehicle alone. Moreover, in a model of muscle injury, GDF11 treatment of aged mice 28 days before injury and continued for 7 days thereafter restored more youthful profiles of myofiber caliber in regenerating muscle. Aged mice treated with GDF11 also showed increased average exercise endurance and grip strength.

In this last paper, they also found that in vitro exposure of aged satellite cells to GDF11, but not myostatin (another member of the TGF-β superfamily) or TGF-β1, produced dose-responsive increases in satellite cell proliferation and differentiation, suggesting that GDF11, in contrast to myostatin, can act directly on satellite cells to alter their function.

3 A tale of surprise and new drugs

The results about the role of GDF11 in the rejuvenation of muscles were not without effect. The surprise came from the prior knowledge that myostatin, a close related protein to GDF11, was known to reduce myogenesis [31]. Indeed, a more recent paper has denied the results obtained by those investigations. Egerman et al. [32] carefully re-assessed this hypothesis and discovered that the previously used reagents to detect GDF11 were nonspecific, i.e. the antibodies could not distinguish between myostatin and GDF11. It also appeared that the total levels of myostatin/GDF11 actually increase with age, contradicting the prior reports [28, 30]. A more specific assay for GDF11 showed a trend towards increased GDF11 levels in serum from older rats and humans compared to younger individuals. Moreover, GDF11 mRNA levels rose in rat muscle with increased age. In vitro experiments also showed that both GDF11 and myostatin induce the same signaling pathways (SMAD 2/3 and MAPK activation) at similar degrees in primary and immortalized human skeletal muscle cells and that differentiation of human primary myoblasts into myotubes was inhibited by GDF11 and myostatin. By using the same treatment protocol established by Sinha *et al.* [30], these authors found no differences in the regenerative capacity of skeletal muscle of aged mice treated with GDF11 or vehicle at the 7-day time point post-cardiotoxin injury. Rather, higher systemic levels of GDF11 were associated with impaired regeneration in young mice, as indicated by a greater number of very small myofibers in the GDF11-treated muscles. Finally, they also showed that treatment with GDF11 decreased the growth of adult and aged satellite cells cultures in a dose-dependent manner. Consistent with the demonstration in the present study that GDF11 increases with age, the same group had previously demonstrated that the downstream myostatin/GDF11 signaling pathway, characterizedby SMAD3 phosphorylation, was also elevated in the aged rat [31]. The implication of this study is that if old individuals are found with very high levels of GDF11, and this is coincident with muscle loss (sarcopenia), they are candidates for either GDF11-specific blockade or for a more general blockade of the GFD11, myostatin, and their receptors.

Although at first glance the data generated by Egerman and colleagues seemed to conflict with Amy Wagers team's results, there could be multiple forms of GDF11 and only one could decrease with age, as reported by *The Scientist* in an e-mail correspondence with Amy Wagers [33]. Moreover, the Novartis group injured the muscle more extensively and then treated it with more GDF11 than Wagers' group had done, so the results may not be directly comparable (the Novartis team used young animals and a dose of GDF11 three times higher). The fact is that the results published by Egerman and colleagues could help to explain the mechanism behind bimagrumab, an experimental Novartis treatment for muscle weakness and wasting [34]. The drug, which is currently in clinical trials, blocks myostatin — and perhaps GDF11 as well [35].

In brief, it is not doubted that young blood renews old mice, but Novartis team says that the Harvard group's explanation is wrong. Probably the truth stays in the middle, and maintaining GDF11 levels in an appropriate physiological range would be essential for muscle health. It is also important to recall what Amy Wagers said: "We're not de-ageing animals. We're restoring function to tissues" [36]. Alternatively, other factors may act in this context. In 2014, Irina and Michael Conboy identified [37] one of the anti-aging factors circulating in the blood: oxytocin, a nonapeptide produced by hypothalamus, which is involved in parturition and bonding. They observed that oxytocin levels declined in old mice (18-24 months), and when injected subcutaneously into aged mice, oxytocin recovered the regenerating capacity of muscle cells upon cardiotoxin injury.

In the field of identification of specific rejuvenation factors, there are still many unresolved issues. For example, how CCL11 or GDF11 improve aged tissue specific stem cell microenvironment are largely undetermined. Moreover, some observations are difficult to be reconciled one with the other. For example, the blood from aged individuals, which reduces GDF11 in the serum [28], negatively affects neurogenesis and cognitive function in young individuals [27]. This is in contradiction with the fact that the old serum is diluted in young serum, calling in question whether microRNAs (miRNAs), or ther protein factors, could target the GDF11 pathway in order to increase or decrease its expression. What is relevant to our review is that on the basis of Wagers' results, at least one company is attempting to replicate the effect in humans using blood plasma from healthy young people to treat patients with Alzheimer's disease. It is interesting to recall that in 1972, two researchers at the University of California studied the lifespans of old–young rat pairs. Older partners lived for four to five months longer than controls, suggesting for the first time that circulation of young blood might affect longevity [38]. In September 2014, a start-up company, Alkahestin Menlo Park, California, began an open label, single group assignment clinical trial assessing the safety and efficacy of 1 unit of plasma from young donors (males, aged 30 or younger) to treat mild-to-moderate Alzheimer's disease (ClinicalTrials.gov Identifier: NCT02256306). Results concerning the primary (symptoms and adverse

events) as well as secondary outcomes (MRI and blood biomarkers) are awaited in this year. However, although this trial is designed over 4 weeks, concerns of this practice may arise: for example, the possibility that activation of stem cells over a long period of time would result in an increase in cancer incidence.

4 Pros and cons of whole blood versus specific factors

The advantage of giving young blood to an old person is that it may contain different rejuvenating factors that could have pleiotropic effects on many diseased organs at the same time. Indeed, whole blood is a mixture of cells, colloids and crystalloids. Each of these components can be separated from the others in order to obtain packed red blood cell (PRBC) concentrate, platelet concentrate, fresh frozen plasma and cryoprecipitate, used in different indications [39]. The transfusion of whole blood or its components is a safe clinical practice today and has many applications in regenerative medicine [40]. However, some of its components, such as plasma proteins, leucocytes, red cell antigens, plasma and pathogens, may give rise to adverse effects that may range from mild allergic manifestations to fatal reactions [39]. Moreover, we do not know for humans if, for example, plasma from a young donor contains factors beneficial to patients with muscular dystrophy or Alzheimer's. For these reasons, the identification of specific factors helping specifically old organs and tissue to rejuvenate or heal may be a safer approach. Rando, Wagers and other would prefer to see testing for a specific blood factor or combination of known factors synthesized on the bench in the lab [36]. The disadvantage in this case is that the mechanism of action of identified factors (CCL11, GDF11, or oxytocin) is far to be fully understood. It can also be that these factors do not act directly, but they do depend on epigenetic mechanisms (e.g, miRNAs), or that other elusive blood components acts with them to compound their rejuvenation effects.

5 Conclusions

This is a tale of long search by human beings of the 'fountain of youth'. Parabiosis has suggested over a long period of time that factors from young blood may help diseased or aged tissues to regenerate. Some would prefer to administer whole blood or its derivatives, such as plasma, while others are more akin to deliver specific factors or cocktails of factors. The best scenario would be to use patient's own plasma or platelet-derived cytokines and growth factors to stimulate wound healing and tissue regeneration. Some hints are coming out from animal studies, but the link to humans is still to be found.

Conflict of interest statement: Authors state no conflict of interest

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