ARTICLES

Systemic signals regulate ageing and rejuvenation of blood stem cell niches

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Ageing in multicellular organisms typically involves a progressive decline in cell replacement and repair processes, resulting in several physiological deficiencies, including inefficient muscle repair, reduced bone mass, and dysregulation of blood formation (haematopoiesis). Although defects in tissue-resident stem cells clearly contribute to these phenotypes, it is unclear to what extent they reflect stem cell intrinsic alterations or age-related changes in the stem cell supportive microenvironment, or niche. Here, using complementary *in vivo* and *in vitro* heterochronic models, we show that age-associated changes in stem cell supportive niche cells deregulate normal haematopoiesis by causing haematopoietic stem cell dysfunction. Furthermore, we find that age-dependent defects in niche cells are systemically regulated and can be reversed by exposure to a young circulation or by neutralization of the conserved longevity regulator, insulin-like growth factor-1, in the marrow microenvironment. Together, these results show a new and critical role for local and systemic factors in signalling age-related haematopoietic decline, and highlight a new model in which blood-borne factors in aged animals act through local niche cells to induce age-dependent disruption of stem cell function.

Age-associated pathologies represent a significant and growing global health care concern, particularly as demographic trends predict a doubling in the number of individuals over 65 years of age in the next 20 years[1]. In the haematopoietic system, ageing is associated with deficient immune function and increased incidence of malignancy, particularly of the myeloid subtype[2]. Age-associated blood diseases are thought to arise in part owing to discrete changes in aged haematopoietic stem and progenitor cells (HSPCs), including a considerable expansion of HSPCs in aged bone marrow, coupled paradoxically with a reduced capacity for blood reconstitution and skewed differentiation potential after transplant[2–5]. Previous work clearly demonstrates cell-intrinsic alterations (for example, DNA damage, oxidative stress, and senescence-associated protein induction) that are associated with and probably contribute to HSPC ageing[2,3,6–8]; however, other studies also indicate a role for non-autonomous signals in this process. In particular, the ability of anatomically defined stromal elements, or 'niches', within the bone marrow to regulate HSPC function[9–11] suggests that changes in extrinsic inputs may also contribute markedly to age-dependent haematopoietic dysfunction. Moreover, given the simultaneous effect of ageing on several organ systems, it is possible that global alterations in systemic tissue regulators further modulate HSPC function in aged animals and perhaps coordinate ageing across tissues[12,13].

Here we investigate the possible role of local microenvironmental and systemic factors in HSPC ageing, using direct isolation of haematopoietic stem cell (HSC)-regulatory niche cells and an *in vivo* parabiotic mouse system to assess age-related HSPC phenotypes that may be modulated extrinsically. These studies clearly demonstrate that HSPC-regulatory niche cells undergo age-induced alterations in their ability to support HSPC function, and that these age-related changes in niche activity can be reversed by systemic factors. Systemic modulation of stem cell–niche cell interactions may thus provide promising, new avenues for restoring aged tissue function.

Systemic signals can rejuvenate aged HSCs

To assay the effects of age-regulated systemic factors on HSPC number and function, we generated heterochronic parabiotic pairs, in which young mice (2 months) were surgically joined to aged partners (>21 months), and compared these to isochronic pairs (young–young or aged–aged) joined at identical ages. Parabiosis generates animals that share a common blood circulation, and thereby tests specifically whether physiological levels of circulating cells or factors can significantly alter tissue function[14–17]. Parabiotic pairs were maintained 4–5 weeks before analysis, and congenic markers were used to distinguish haematopoietic cells from aged ($CD45.2^+$) versus young ($CD45.1^+$) partners[18].

Consistent with previous studies[18], parabiosis itself had no apparent effect on HSPCs; isochronic parabionts exhibited precursor frequencies and numbers equivalent to age-matched controls that were not joined (Fig. 1a, Supplementary Fig. 1a and data not shown). The frequencies and numbers of primitive long-term reconstituting HSCs (LT-HSC, $c\text{-}kit^+Lin^-Sca\text{-}1^+CD34^-Flk2^-$ ($KLS34^-Flk2^-$), Supplementary Table 1 and refs 19, 20) were similarly unchanged in young-heterochronic partners exposed to an aged circulatory system (Fig. 1a and Supplementary Fig. 1a). In contrast, aged-heterochronic partners showed significant recovery of LT-HSC frequency and number, which approached normal 'youthful' levels (Fig. 1a and Supplementary Fig. 1a). Notably, these effects arose from changes in the aged HSC population itself (as opposed to trafficking of 'young' cells ($CD45.1^+$) to the aged partners' marrow), as we specifically distinguished HSCs from aged ($CD45.2^+$) versus young ($CD45.1^+$) partners by flow cytometry.

Paralleling recovery of LT-HSC frequency and number, heterochronic parabiosis also induced recovery of LT-HSC function in aged mice. Despite increased HSC frequencies in aged marrow, aged LT-HSCs typically exhibit impaired haematopoietic engraftment, manifested by reduced reconstitution of peripheral blood leukocytes, and a differentiation bias that favours myeloid over B lymphoid development (Fig. 1c and ref. 3). However, bone marrow cells from aged mice exposed to young systemic factors by heterochronic parabiosis (Supplementary Fig. 1c) showed a profound recovery of engraftment potential (Fig. 1c; total leukocytes), as well as restoration of youthful ratios of B lymphoid to myeloid cells (Fig. 1c). Thus, accumulation

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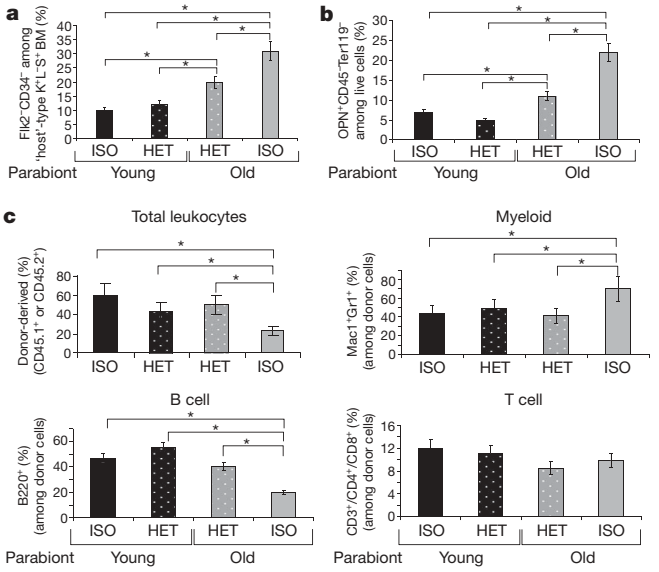

Figure 1 | Exposure to young circulating factors restores LT-HSC numbers and function, and osteoblastic niche cell number in aged mice. Bone marrow or bone cells were isolated from the young or old partner of heterochronic (HET) or isochronic (ISO) control parabionts (parabiont denotes one mouse of a parabiotic pair). The frequency of 'host'-type LT-HSC (percentage of $Flk2^-CD34^-$ cells among c-$kit^+Sca\text{-}1^+$ cells previously gated on live Lin^- cells and expressing the relevant congenic marker ($CD45.2^+$ for aged animals and $CD45.1^+$ for young heterochronic partners)) and osteoblastic niche cells (percentage of $OPN^+CD45^-Ter119^-$ among live cells) were determined by FACS and used to determine total number (Supplementary Fig. 1). **a**, **b**, Frequencies of endogenous LT-HSCs (**a**) and osteoblastic niche cells (**b**) are shown for the 4 long bones, and represent 5–7 independent experiments, each including 2–4 pairs of mice per condition. Data are presented as mean ± s.d.*$P < 0.05$ by two-way ANOVA. **c**, Long-term, multi-lineage reconstitution of irradiated recipients by donor bone marrow cells collected from heterochronic or isochronic young or aged mice was evaluated by FACS 12 weeks after transplant. The frequency ± s.d. of donor-derived ($CD45.2^+$ for reconstitution by bone marrow cells from isochronic pairs or heterochronic-aged mice, or $CD45.1^+$ for bone marrow cells from heterochronic-young mice) mature blood cells was calculated among total peripheral blood cells or after staining peripheral blood with markers of myeloid (Mac-1, Gr-1), B (B220) or T (CD4, CD8, CD3) lymphoid cells. Data are averaged from three independent experiments. *$P < 0.05$ by Student's *t*-test.

and altered lineage potential of aged HSCs can be substantially ameliorated by exposure to a young systemic environment.

Ageing of HSC-regulatory niche cells

Co-localization studies implicate several cell types as components of HSC niches, including endothelial, reticular and osteoblast cells[21]; however, functional analyses demonstrate a particular, direct role for bone-forming osteoblasts in controlling HSC numbers and activity[10,11,22]. We previously developed a strategy to isolate mouse osteoblasts prospectively, using the cell surface markers $OPN^+CD45^-Ter119^-$ (ref. 9 and Supplementary Fig. 2), and showed using a pharmacological mobilization model that these cells directly communicate physiologically appropriate changes in HSC activity[9]. Thus, because $OPN^+CD45^-Ter119^-$ cells are sufficient to recapitulate functionally relevant HSC regulation[9], we refer to them as 'osteoblastic niche cells', and used this direct isolation strategy to investigate how niche cells affect ageing and rejuvenation of HSCs.

Osteoblastic niche cells were isolated using fluorescence-activated cell sorting (FACS) from collagenase-treated bones of young or aged individual mice (Supplementary Fig. 2), or isochronic parabionts (Fig. 1b and Supplementary Fig. 1b). Notably, cells sorted from aged mice maintained enriched expression of osteoblast-associated markers and *in vitro* bone-forming capacity equivalent to cells from young mice (Supplementary Fig. 2c–e). However, similar to HSCs[2,3], both the frequency and total number of osteoblastic niche cells isolatable from aged mice were increased up to fourfold compared to young controls (Fig. 1b and Supplementary Figs 1b and 2).

This expanded pool of aged osteoblastic niche cells also acquired functional alterations affecting their interactions with HSCs. We exposed young lineage-negative (Lin^-) bone marrow cells[17] to osteoblastic niche cells sorted from either young or aged mice (Supplementary Fig. 3a). After short (36 h) *ex vivo* exposure, significantly more HSPCs ('KTLS' cells, Supplementary Table 1 and refs 14, 23) were recovered from cultures containing aged osteoblastic niche cells than from equivalent cultures containing young niche cells (Supplementary Fig. 3b, c). Thus, interaction of young HSCs with aged osteoblastic niche cells is sufficient to induce HSPC accumulation reminiscent of that seen normally in aged marrow[3], raising the possibility that the HSC rejuvenating effects of heterochronic parabiosis (Fig. 1 and Supplementary Fig. 1) occur indirectly—by reverting age-related changes in osteoblastic niche cells.

Systemic signals restore aged niches

To test this hypothesis, we next determined whether age-dependent alterations in osteoblastic niche cell number or function could be corrected by heterochronic parabiosis. Similar to the effects of heterochronic parabiosis on HSCs, osteoblast frequency and number were restored to youthful levels when aged animals experienced a young systemic environment (Fig. 1b and Supplementary Fig. 1b; $P = 0.03$ and $P = 0.019$, respectively, by two-way analysis of variance (ANOVA)). Moreover, in contrast to niche cells isolated from aged-isochronic parabionts, niche cells from aged-heterochronic parabionts showed a significantly reduced capacity to cause HSPC accumulation (Fig. 2a, b; $P = 0.042$ by two-way ANOVA).

Interestingly, these experiments also suggested a reciprocal effect of the aged circulatory environment on niche activity in young-heterochronic partners. Although heterochronic parabiosis did not alter the frequency or number of niche cells in young mice joined to aged partners (Fig. 1 and Supplementary Fig. 1), osteoblastic niche cells isolated from young-heterochronic parabionts did induce a slight expansion of young HSPCs as compared to niche cells from young-isochronic parabionts (Fig. 2b; $P = 0.047$ by two-way ANOVA). These data probably indicate the presence of 'ageing' factors in the circulation of older animals that actively alter niche function, but might also reflect a dilution of youthful 'rejuvenating' factors in heterochronic parabionts. Regardless, these studies clearly demonstrate that exposure to a young systemic environment is sufficient to reverse the *in vivo* expansion of aged osteoblastic niche cells and restore their ability to appropriately regulate HSPC number.

Local niche cells regulate HSC ageing

To test whether defects in the engraftment and differentiation of aged HSCs might, similar to HSC number, be controlled by alterations in niche cell activity, we next compared the ability of young Lin^- HSPCs to reconstitute haematopoiesis in lethally irradiated, CD45-congenic animals after brief (36 h) exposure to osteoblastic niche cells isolated from isochronic-young, isochronic-old, heterochronic-young or heterochronic-old parabionts (Fig. 2c). Notably, similar to the impaired engraftment function of naturally aged HSCs[3], young HSPCs exposed *in vitro* to aged-isochronic niche cells showed a reduced capacity for haematopoietic reconstitution as compared to young HSPCs exposed to young-isochronic niche cells (Fig. 2d; $P = 0.037$ by Student's *t*-test). Young HSPCs exposed to aged niche cells also exhibited biased differentiation potential (myeloid cell overproduction, $P = 0.044$ by Student's *t*-test, and B-cell underproduction, $P = 0.026$ by Student's *t*-test; Fig. 2d) similar to that typically observed for aged HSCs[3]. Thus, interaction with aged osteoblastic niche cells is sufficient to induce defects in HSC function that mirror physiological ageing of HSCs[3].

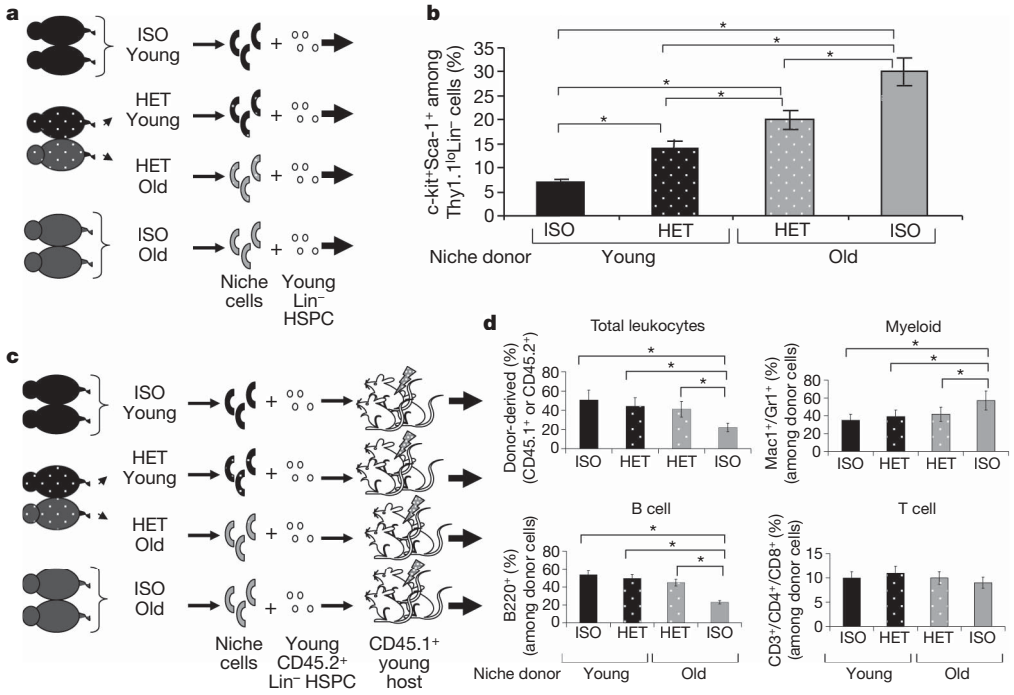

Figure 2 | Circulating factors rejuvenate HSC regulatory activity of osteoblastic niche cells. a, Osteoblastic niche cells were isolated and pooled from young or old partners of heterochronic or isochronic parabiosis. Two-thousand osteoblastic cells were cultured with 100,000 Lin^- HSPCs isolated from young donor mice. **b**, After 36 h, the frequency of KTLS HSPCs was determined by FACS. Data are presented as the mean percentage ± s.d. of c-$kit^+Sca\text{-}1^+$ cells among $Thy1.1^{lo}Lin^-$ cells previously gated to exclude dead cells. Data are averaged from four independent experiments, each including 1–4 pairs of mice in each condition. $^*P < 0.05$ by two-way ANOVA. **c**, Osteoblastic niche cells were isolated and pooled from young or old heterochronic partners and co-cultured with Lin^- HSPCs as in **a**. After 36 h, cultured cells (donor; $CD45.2^+$) were transplanted into lethally irradiated mice (host; $CD45.1^+$). **d**, Long-term multi-lineage reconstitution of mature leukocytes by donor cells was evaluated by FACS at 16 weeks post-transplant. Data are presented as the frequency (mean ± s.d.) of donor-derived ($CD45.2^+$) mature blood cells among total peripheral blood cells (top left), or of myeloid ($Mac1^+/Gr1^+$; top right), B ($B220^+$; bottom left) or T ($CD4^+/CD8^+/CD3^+$; bottom right) cells among donor-derived ($CD45.2^+$) leukocytes. Data are averaged from five independent experiments, each including triplicate co-cultures for each experimental condition. $^*P < 0.05$ by Student's *t*-test.

In contrast to the defects in HSC function induced by aged-isochronic niche cells, aged-heterochronic niche cells did not alter the reconstituting activity of young HSCs. In fact, congenic recipients transplanted with young HSPCs exposed to osteoblastic niche cells from aged-heterochronic parabionts showed equivalent donor cell engraftment and similar myeloid versus B lymphoid differentiation as animals transplanted with HSPCs exposed to young osteoblastic niche cells (Fig. 2d). Animals transplanted with young HSPCs exposed to osteoblastic niche cells from young-heterochronic parabionts also showed a youthful pattern of haematopoietic engraftment (Fig. 2d). Notably, this effect of heterochronic parabiosis on aged osteoblastic niche cells represents a direct effect on endogenous, 'aged' cells, as $OPN^+CD45^-Ter119^-$ cells do not cross-engraft in the bones of parabiotic pairs (S.R.M. and A.J.W., unpublished observations). Together, these data demonstrate that age-induced, functional alterations in HSC-regulatory niche cells, which are sufficient to induce ageing phenotypes in young HSCs, can be reversed by young circulating factors. These findings further suggest that the rejuvenating effects of a young circulation on HSCs (Fig. 1 and Supplementary Fig. 1) are communicated indirectly, by signalling from rejuvenated osteoblastic niche cells.

Mechanisms of HSPC and niche-cell ageing

To begin to uncover the mechanisms underlying systemic regulation of osteoblastic niche cells, we next developed an *in vitro* model of heterochronic parabiosis, in which niche cells from young mice were exposed *ex vivo* to serum from old mice (Fig. 3a, b) or, conversely, niche cells from old mice were exposed to serum from young mice (Fig. 3c, d). After 18–24 h, the cells were washed extensively, and the effect of this heterochronic serum exposure on niche activity was tested in short-term co-culture assays with young mouse HSPCs (which were never directly exposed to aged or young sera). Notably, when compared to young niche cells previously exposed to young serum, or to control cultures containing fetal bovine serum (FBS), young osteoblastic niche cells previously exposed to aged serum enhanced the accumulation of young HSPCs—to levels resembling those seen after direct exposure of young HSCs to physiologically aged osteoblastic niche cells (Fig. 3c; $P = 0.036$ compared to young serum, and $P = 0.023$ compared to FBS, by one-way ANOVA; also compare to Fig. 2b and Supplementary Fig. 3). Likewise, when young niche cells were pre-exposed to serum from aged human donors (>69 years), these cells induced accumulation of young HSPCs (Supplementary Fig. 4). Thus, previous exposure of niche cells from young mice to serum from old mice or humans recapitulates the effects of aged niche cells on accumulation of young, phenotypically defined HSCs. In contrast, aged osteoblastic niche cells pre-exposed to young serum (Fig. 3b) showed a reduced capacity to induce accumulation of LT-HSCs, as compared to control cultures in which aged osteoblastic niche cells were instead pre-exposed to aged serum (Fig. 3d; $P = 0.0169$ by one-way ANOVA). Together, these experiments establish an *in vitro* system that at least partially recapitulates the *in vivo* effects of systemic factors on osteoblastic niche cells, and further demonstrates that these effects are conserved in mice and humans.

To investigate how age-dependent changes in osteoblastic niche cells trigger subsequent changes in HSCs, we next examined the survival and transcription profiles of HSCs exposed to young or old osteoblasts. LT-HSCs ($KLS41^-48^-SLAM^+$) cultured with young versus aged osteoblastic niche cells showed no significant differences in apoptosis (Supplementary Fig. 5a, b; $P = 0.05982$ by Student's

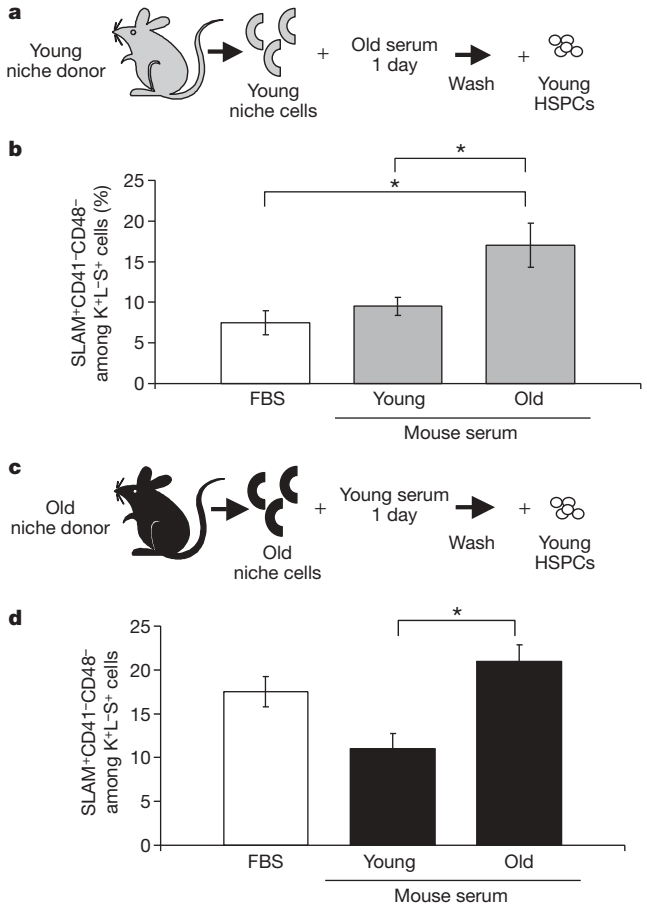

Figure 3 | Heterochronic serum alters osteoblastic niche cell activity. **a**, **c**, Cartoons showing the niche activity assays used to detect ageing (**a**) or rejuvenation (**c**) of osteoblastic niche cells. Osteoblastic niche cells were isolated from young (2 months old) (**a**) or aged (22 months old) (**c**) mice, and 2,000 niche cells were cultured in media supplemented with FBS, or serum from young (2 months) or aged (21 months) mice. After 24 h, media containing FBS, or young/aged mouse serum was removed, and osteoblasts were washed with serum-free media before addition of 100,000 young HSPCs (Lin^- bone marrow cells). Co-cultures were maintained for a further 24 h in FBS-supplemented media. **b**, **d**, Frequencies of LT-HSCs were determined by FACS for c-$kit^+Lin^-Sca\text{-}1^+CD41^-CD48^-SLAM^+$ ($KLS41^-48^-SLAM^+$) cells[33], and represent the mean percentage ± s.e.m. of $CD41^-CD48^-SLAM^+$ cells among c-$kit^+Sca\text{-}1^+$ cells previously gated on live Lin^- cells. Data represent triplicate wells per experimental condition for $n = 4$ (**b**) or $n = 3$ (**d**) experiments. $^*P < 0.05$ by one-way ANOVA.

t-test); however, they did exhibit altered gene expression. Recent profiling studies of young versus aged LT-HSCs showed that age-dependent skewing of HSC fate potential involves concurrent suppression of lymphoid specification genes and induction of myeloid specification genes in HSCs[3]. Focusing on a subset of messenger RNAs previously found to undergo such age-dependent alterations in expression by LT-HSCs[3], we performed real-time PCR analysis on FACS-sorted young LT-HSCs that had been exposed to aged or young osteoblastic niche cells from isochronic or heterochronic parabionts. Exposure to isochronic-aged niche cells significantly increased expression by young LT-HSCs of several age-regulated myeloid markers (*Pml*, *Fli1* and *Runx1*) and decreased expression of lymphoid markers (*Il7* and *BAFF* (also known as *Tnfsf13b*)), as compared to expression profiles in identical LT-HSCs exposed instead to isochronic-young osteoblastic niche cells (Supplementary Fig. 5c). Likewise, several stem-cell-regulatory genes (*Sox4*, *Notch1* and *Notch2*) previously reported to exhibit differential expression in young versus aged HSCs[3] showed altered expression in young LT-HSCs exposed to aged osteoblastic niche cells (Supplementary Fig. 5c). Notably, these niche cell-induced effects on gene expression were not observed when LT-HSCs were exposed to aged osteoblasts that had been functionally restored by heterochronic parabiosis (Supplementary Fig. 5c). These data correlate directly with the alterations in haematopoietic reconstitution and lineage potential seen after HSC exposure to isochronic-aged or heterochronic-aged niche cells (Fig. 2c, d), and implicate specific effects on HSC gene expression as a mechanism by which exposure to aged osteoblasts impairs young HSC function.

Local IGF-1 regulates niche cell function

As noted earlier, age-dependent dysfunction of tissue-specific progenitors occurs in many organs, including blood, brain, liver and skeletal muscle[2]. In muscle, these age-dependent deficits can be ameliorated by enforced expression of insulin-like growth factor-1 (IGF-1), an evolutionarily conserved ageing and longevity regulator[24]. IGF-1 is also a potent anabolic agent for bone[25,26], and can regulate lineage choices of differentiating HSPCs[27,28]. In light of these relevant IGF-1 activities, we decided to test whether serum factors in aged mice affect IGF-1 activity in the marrow and, in turn, influence age-dependent niche cell–HSC interactions. We exposed young HSPCs to young or aged osteoblastic niche cells pre-incubated with neutralizing antibody against IGF-1 or with isotype control antibody (Fig. 4a). Notably, pre-exposure of aged osteoblastic niche cells to anti-IGF-1 antibody (10 μg ml^{-1}; 12–18 h) completely abolished the accumulation of phenotypically defined LT-HSCs typically seen in response to these cells (Fig. 4a). Likewise, IGF-1 neutralization during pre-exposure of young niche cells to aged serum disrupted the ability of aged serum to condition young niche cells to induce HSC accumulation (Fig. 4b). In contrast, IGF-1 neutralization had negligible effects on the interactions of young HSCs with young niche cells (Fig. 4a), or with young niche cells exposed to young serum (Fig. 4b). IGF-1 neutralization also did not affect HSC frequency or number in control cultures containing HSPCs alone (data not shown). Thus, inhibition of IGF-1 signalling in aged osteoblastic niche cells promotes youthful HSC-regulatory function, indicating that IGF-1 responses in aged osteoblastic niche cells impair their appropriate regulation of HSCs, and thereby contribute to age-associated haematopoietic dysfunction.

To determine whether local or systemic changes in IGF-1 activity underlie these effects on osteoblastic niche cells, we next neutralized IGF-1 signalling *in vivo* by administering anti-IGF-1 systemically (by intraperitoneal injection; Supplementary Figs 6 and 7), or locally (by direct injection into the bone marrow cavity; Supplementary Fig. 6 and Fig. 5a, b). Notably, *in vivo* neutralization of IGF-1 effectively decreased IGF-1 bioactivity, and these effects remained anatomically compartmentalized (Supplementary Fig. 6). Anti-IGF-1 treatment did not alter the function of young osteoblastic niche cells in any of the conditions tested (Fig. 5b and Supplementary Fig. 7); however, local IGF-1 neutralization, by antibody delivery directly into the bone marrow, caused a marked decrease in the capacity of subsequently isolated aged osteoblastic niche cells to promote HSC accumulation in co-culture assays. This effect was dose-dependent and significant (Fig. 5b, $P = 0.040$, $P = 0.035$ and $P = 0.012$ compared to uninjected contralateral leg, or to control mice injected with PBS or isotype control, respectively, by two-way ANOVA). In contrast, systemic administration of anti-IGF-1 had no significant effect on the HSC-regulatory activity of aged osteoblastic niche cells (Supplementary Fig. 7). Thus, local, not systemic, IGF-1 seems to induce ageing of HSC-regulatory niche cells, and neutralization of IGF-1 signalling in the bone marrow microenvironment reverts age-related changes in osteoblastic niche cells that impair their appropriate regulation of HSCs.

Conclusions

The studies reported here show a new role for the stem cell niche in initiating age-related dysfunction of tissue-specific stem cells and in rejuvenating age-dependent stem cell defects. We find that alterations in aged osteoblastic niche cells relay ageing phenotypes to

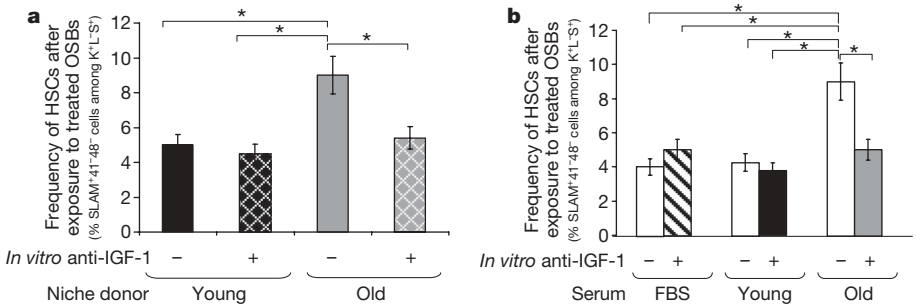

Figure 4 | Neutralization of IGF-1 *in vitro* inhibits accumulation of HSPCs induced by aged osteoblastic niche cells. a, Osteoblasts were isolated from young (2 months old) or aged (22–24 months old) mice. Niche cells (2,000) were cultured in media (+FBS) containing 10 $\mu g\,ml^{-1}$ isotype control antibody (−) or anti-IGF-1 neutralizing antibody (+). After 24 h, media was removed and osteoblasts were washed with serum-free media before adding 100,000 young Lin^- HSPCs. Co-cultures were maintained for a further 24 h in media (+FBS). The frequency of LT-HSCs ($KLS41^-48^-SLAM^+$)[33] was determined by FACS. Data are presented as mean frequency ± s.e.m. of $CD41^-CD48^-SLAM^+$ cells among c-$kit^+Sca\text{-}1^+$cells previously gated on live Lin^- cells. $n = 3$ independent experiments, each condition analysed in triplicate. $^*P < 0.05$ by Student's *t*-test. **b**, Young osteoblastic niche cells (2,000) were cultured in media (+FBS) or serum from young (2 months old) or aged (21 months old) mice, together with 10 $\mu g\,ml^{-1}$ isotype control antibody (−) or anti-IGF-1 neutralizing[26,34,35] antibody (+). After 24 h, media containing young or aged serum (±antibodies) was removed and osteoblasts were washed with serum-free media before the addition of 100,000 young Lin^- HSPCs. Co-cultures were maintained for a further 24 h in FBS-containing media, and the frequency ± s.e.m. of primitive LT-HSCs was determined by FACS. Data represents three independent experiments, as in **a**. $^*P < 0.05$ by Student's *t*-test.

HSPCs, and that the effects of ageing on niche cells can be modulated by blood-borne factors. We therefore suggest that while under youthful conditions osteoblastic niche cells promote homeostatic stem-cell maintenance, these niche cells are altered by ageing such that they instead allow the enhanced accumulation of (dysfunctional) HSCs (Supplementary Fig. 8). Because aged osteoblastic niche cells alone, without any other environmental inputs, are sufficient to induce HSC accumulation *in vitro*, we propose that age-related changes in these niche cells contribute directly to the changes in HSC frequency, number and function seen in aged mice. These age-specific alterations in osteoblastic niche cells seem to be signalled by as yet uncharacterized circulating factors that act in part by altering IGF-1 signalling in the niche cells themselves. Although the precise identity of these age-altered systemic regulators of IGF-1 remains unclear, recent linkage of age-associated pathologies in the skin, bone, skeletal muscle and haematopoietic system with systemically regulated Wnt signalling[29,30] may implicate direct interaction or co-regulation of these pathways in the systemic regulation of several aged stem cell populations[31]. Regardless, the particular effect, seen here, of IGF-1 neutralization in restoring youthful function to aged osteoblastic niche cells highlights a new and important activity for this well-studied growth factor in controlling the regulation of stem cells by their niches. This role of IGF-1 in osteoblastic niche cells is consistent with studies in *Caenorhabditis elegans* and other model organisms, which emphasize the age-promoting effects of IGF-1 signalling[32], but contrasts with reports in skeletal muscle indicating that induced local expression of IGF-1 maintains regenerative capacity in aged animals[24]. Thus, IGF-1 seems to regulate ageing tissue function in a complex and tissue-specific manner, demonstrating the importance of contextual signalling in the physiological activity of regulatory growth hormones. Considered therapeutically, the ability of age-regulated circulating factors to reversibly modulate haematopoietic activity by local, IGF-1-mediated effects on stem cell niches (Supplementary Fig. 8) provides impetus for developing new strategies that target the circulatory environment to maintain proper function of

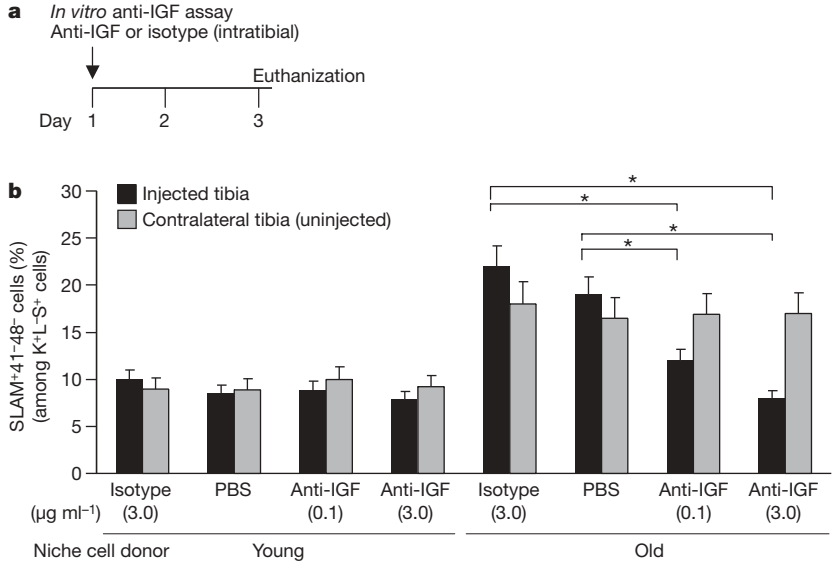

Figure 5 | Local neutralization of IGF-1 in the bone marrow inhibits accumulation of HSPCs induced by aged osteoblastic niche cells. a, Young or old mice were treated (day 1) with either 0.1 or 3.0 $\mu g\,ml^{-1}$ neutralizing anti-IGF antibody by intratibial injection into one leg (injected). Osteoblastic niche cells were collected from the injected or uninjected tibia of euthanized mice on day 3, washed, and co-cultured with untreated young HSPCs (Lin^- bone marrow cells). **b**, After 24 h, the frequency ± s.d. of LT-HSCs ($KLS41^-48^-SLAM^+$) was determined, as in Fig. 4a. Control mice were treated with isotype antibody, PBS, or left untreated (data not shown). Co-cultures were performed in duplicate or triplicate for each condition in each experiment ($n = 3$). $^*P < 0.05$ by two-way ANOVA.

niche cells and stem cells to extend the youthful function of the ageing blood system.

METHODS SUMMARY

Mice, antibodies, flow cytometry and bone marrow isolation. Aged (18–22 months old) C57Bl/6 mice were from the National Institute on Aging (NIA), JAX or Taconic; young (2 months old) C57Bl/6 ($CD45.1^{-}CD45.2^{+}$) mice and congenic B6.SJL ($CD45.1^{+}CD45.2^{-}$) mice were from JAX or Taconic. Mice were joined in parabiosis for 4–5 weeks before analysis. Details of cell isolation and flow cytometry, with associated references, are provided in Methods.

Osteoblast isolation, short-term culture, cell transplant and IGF-1 neutralization. Osteoblasts were isolated from the bones of parabiotic mice by enzymatic digestion and FACS[9]. Sorted populations of osteoblastic niche cells were tested for HSC regulatory activity by *in vitro* co-culture with FACS-isolated HSPCs. HSC frequency and number after exposure to osteoblastic niche cells isolated from isochronic or heterochronic parabionts were evaluated by flow cytometry (details in the text and Methods). HSC function was measured by long-term haematopoietic reconstitution after transplantation into lethally irradiated (950 rad) congenic mice. Where indicated, young or aged niche cells were pre-incubated with neutralizing antibodies against IGF-1 or isotype control antibody before HSPC addition. IGF-1 function was also tested *in vivo* by intraperitoneal or intratibial injection of anti-IGF-1 into young or aged mice.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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METHODS

Mice. Aged C57Bl/6 mice were obtained from the National Institute on Aging (NIA); young C57Bl/6 ($CD45.1^-CD45.2^+$) mice or young B6.SJL ($CD45.1^+CD45.2^-$) mice were obtained from JAX or Taconic, or were bred in the animal care facilities at the Joslin Diabetes Center. Mice were housed under specific pathogen-free conditions, and all procedures were approved by an Institutional Animal Care and Use Committee. Animals used as recipients in transplantation studies ($CD45.1^+$ B6.SJL) were bred and maintained at the Harvard School of Public Health.

Antibodies and flow cytometry. The antibodies used in these studies included HIS51 (anti-Thy1.1, phycoerythrin (PE) conjugate), 2B8 (anti-c-kit, Pacific Blue (PB) conjugate), D7 (anti-Sca-1, Ly6A/E, allophycocyanin (APC) conjugate), A2F10 (anti-FLK2 PE conjugate, RAM34 (anti-CD34, FITC conjugate), A20 (anti-CD45.1, FITC or PE-Cy7 conjugate) 104 (anti-CD45.2, PE or APC-Cy7 conjugate), (anti-SLAM (CD150), PE-Cy7 conjugate; BioLegend) (anti-CD41, PE conjugate), (anti-CD48, PE conjugate), ZH2 (rat anti-osteopontin, unconjugated; Chemicon), rat anti-IgG FITC conjugate), Ter119 (anti-erythrocyte-specific antigen, Ly-76, APC conjugate), and 30-F11 (anti-CD45, APC-Cy7 conjugate). Lin markers included the biotinylated antibodies 145-2C11 (anti-CD3), GK1.5 (anti-CD4), 53-7.3 (anti-CD5), Ter119 (anti-erythrocyte-specific antigen, Ly-76), 6B2 (anti-B220), 8C5 (anti-Gr-1), and M1/70 (anti-Mac-1). Streptavidin PE-TXR was used as the secondary antibody for Lin marker staining. All antibodies were from eBioscience unless otherwise noted. Cells (2×10^6 per 100 μl) were stained with antibodies for 10 min in HBSS supplemented with 2% donor bovine serum (DBS). For Annexin V staining, cells were washed in Annexin V staining buffer (0.01 M HEPES, pH 7.4, 14 M NaCl, 2.5 mM CaCl) after first staining for cell surface markers ($KLS41^-48^-SLAM^+$), and then incubated with Annexin-V-specific antibody (5 μl per 100 μl) for 15 min. Propidium iodide was used as a marker for cell viability and added to all samples just before sample acquisition. Flow cytometric experiments were performed using an LSRII, FACS-Aria (BD Biosciences) or Moflo (Dako). Data were analysed with FlowJo software (Treestar) and represented as histograms, contour or dot plots of fluorescence intensity.

Bone marrow cell and osteoblast isolation. Total bone marrow cells were isolated from mouse femurs and tibias by flushing with HBSS and 2% FBS. Erythrocytes and debris were removed using ammonium chloride potassium (ACK) lysis buffer and filtering through nylon mesh. Osteoblasts were isolated from marrow-depleted bones as described[9], and identified by positive expression of OPN^+ and exclusion of haematopoietic and red blood cell markers, $CD45^-$ and $TER119^-$, respectively.

Cell culture, IGF-1 neutralization and serum assays. HSPCs (1×10^5) and osteoblasts (2×10^3) were isolated as described and co-cultured in RPMI supplemented with 10% FBS plus 100 U ml^{-1} penicillin and 1 mg ml^{-1} streptomycin for up to 36 h, as indicated for each experiment. For co-culture assays performed with aged or young serum from mice, serum was collected after clotting of blood obtained from the tail vein from either aged (18–22 months old) or young (2 month old) animals. Young and aged human serum were purchased from Lifeblood Medical, Inc. Isolated osteoblasts were first cultured overnight in the presence of 15% aged or young serum, and then thoroughly washed in serum-free media before co-culture with Lin^- HSPCs for a further 24 h[9]. For *ex vivo* IGF-1 neutralization, neutralizing[36,37] anti-IGF-1 antibodies (10 μg ml^{-1}; goat-anti-mouse IGF-1, I2157, Sigma) or isotype control antibodies (10 μg ml^{-1}; goat IgG; I5256, Sigma) were added to cultured osteoblasts at a final concentration of 10 μg ml^{-1} for 12–18 h and then removed by thorough washing before the addition of HSPCs.

Haematopoietic progenitor cell isolation and bone marrow transplantation. HSPCs for co-culture experiments were isolated by FACS as Lin^- bone marrow cells (that is, those lacking expression of a cocktail of biotinylated antibodies (CD3, CD4, CD8, CD5, B220, Ter119, Gr-1 and Mac-1), which mark mature haematopoietic cells)[17]. Cells were collected for transplantation after 36 h, as indicated, and 5,000 HSPCs, isolated on the basis of Lin^- analysis as described earlier, were delivered intravenously by retro-orbital injection to lethally irradiated mice (950 rad) along with 3×10^5 unfractionated whole bone marrow cells. Multi-lineage engraftment (percentage donor-derived mature haematopoietic cells) was monitored by FACS analysis of peripheral blood collected by tail vein at 4–16 weeks after transplantation (data are shown for the 16-week time point). Blood leukocytes were stained with antibodies specific for the myeloid (Mac-1-PE and Gr-1-PE), B cell (B220-Alexa750-APC) and T cell (CD3-, CD4-, and CD8-PE-Cy7 antibody cocktail) compartments (all antibodies from eBioscience), and the frequency of donor-derived cells within each of these populations was determined after exclusion of erythrocytes (by Ter119-PE-Cy5) and dead cells (by propidium iodine).

Parabiosis. Parabiosis was performed exactly as described previously[16]. Cross-circulation was determined in a subset of parabiotic pairs by measuring the frequency of donor-derived blood cells from one partner (CD45.1) in the blood or spleen of the other partner (CD45.2). Mice were euthanized 4–5 weeks after joining. Blood chimaerism at the time of euthanization was typically between 47% and 53%. To identify HSCs in the bone marrow of parabiotic mice and distinguish endogenous versus cross-engrafting cells, bone marrow cells were stained with antibodies against mature lineage markers (as detailed earlier) in combination with c-kit, Sca-1, Flk2, CD34 and either CD45.1 or CD45.2. Congenic CD45.1/CD45.2 markers allowed selective analysis of only the endogenous HSC population and excluded any cross-engrafting, partner-derived cells. LT-HSCs ($KLS34^-$ $Flk2^-$) were identified as cells lacking mature lineage markers (Lin^-) and CD34 ($CD34^-$), but expressing c-kit ($c\text{-}kit^+$) and Sca-1 ($Sca\text{-}1^+$). Dead cells were excluded by propidium iodine. The frequency of LT-HSCs is given as the percentage of $Flk2^-CD34^-$ cells among $c\text{-}kit^+Sca\text{-}1^+$ cells previously gated on Lin^-, live cells and for the relevant congenic marker ($CD45.2^+$ for aged heterochronic animals and $CD45.1^+$ for young heterochronic partners). Total numbers were calculated by multiplying the frequency of $CD45.1^+$ or $CD45.2^+$ LT-HSCs among live bone marrow cells by the total number of bone marrow cells collected.

Bone nodule assay. OPN^+ cells were plated at a density of 10 cells per well in a 96-well plate containing α-MEM supplemented with 15% FBS, 100 U ml^{-1} penicillin and 1 mg ml^{-1} streptomycin. On day 15 of culture, ascorbic acid (20 mg ml^{-1}) and β-glyceraldehyde (50 μM) were added to each well. To detect mineralization of bone nodules (day 30), cell cultures were stained with a 1% silver nitrate solution by von Kossa method as previously described[9].

Quantitative RT–PCR. Equal numbers of $OPN^+CD45^-Ter119^-$, OPN^- cells, or $KLS41^-48^-SLAM^+$ LT-HSCs were isolated as described earlier, and total RNA was prepared by sorting cells directly into TRIZOL reagent (Invitrogen), following the manufacturer's specifications. RNA was treated with DNase I to remove genomic contamination and used for reverse transcription according to the manufacturer's instructions (Superscript II kit, Invitrogen). PCR reactions were performed in an ABI-7000 detection system using SYBR green PCR Core Reagents (Applied Biosystems). PCR amplification was performed in a 10-μl final volume containing 1× SYBR Green PCR buffer, 2 mM $MgCl_2$, 0.5 mM dNTPs, 10 ng of each primer, 0.25 U AmpliTaq Gold, and 1 μl of cDNA templates. β-actin (*Actb*) gene expression was used to normalize the amount of each investigated transcript. Primer sequences and PCR cycling conditions were reported previously[3,9].

***In vivo* neutralization of IGF-1.** For *in vivo* neutralization of IGF-1, young (2 months old) or aged (22 months old) mice were treated with either 0.1 or 3.0 μg ml^{-1} neutralizing anti-IGF-1 antibody, described earlier[36,37]. To assess systemic effects, mice received an intraperitoneal injection on day 1 of either 0.1 or 3.0 μg ml^{-1} anti-IGF-1, and were euthanized on day 3. To test local effects, a separate cohort of young or aged mice received anti-IGF-1 by direct injection into the bone marrow on day 1, and was euthanized on day 3. Osteoblasts were collected from the bones of treated or control mice at the indicated time points, and co-cultured with Lin^- HSPCs collected from untreated mice. Control mice were injected with isotype control antibody (3 μg ml^{-1}; described earlier), PBS, or left untreated.

IGF-1 activity assays. MCF-7 cells were obtained from American Type Tissue Culture (ATCC). Stock cultures of MCF-7 were maintained as described. Cells were passaged every 4–6 days and seeded at 1.75×10^6 per plate in complete growth medium (Minimum essential medium (Eagle) with 2 mM L-glutamine adjusted to contain 1.5 g l^{-1} sodium bicarbonate, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate) and supplemented with 20% FBS alone, 20% FBS plus 0.01 mg ml^{-1} recombinant murine insulin, or 20% serum isolated from treated or control mouse bone marrow or peripheral blood. Confluent cultures, 5–8 days after seeding, were used for IGF-1 activity assays. Cultures were washed once with 5 ml sterile PBS and removed from the culture dish by addition of 3 ml trypsin-EDTA in HBSS. After cells had detached from the plate (5 min at room temperature), trypsin was inactivated by addition of 4 ml PBS containing 0.1% (w/v) soybean trypsin inhibitor. The cells were collected by centrifugation at 329*g* (maximum relative centrifugal force (RCF)) for 3 min, and washed three times with MEM. Cells were seeded at 10,000 per ml of MEM medium in 35-mm diameter culture plates with the indicated concentrations of added growth factor(s) or serum. After an 8-day incubation, with no medium changes or additions, cells were collected and counted using a Cellometer Vision cell counter.

Statistical analysis. Results are expressed as mean ± s.d. or s.e.m., as indicated. Data were analysed using the unpaired two-tailed Student's *t*-test or one- or two-way ANOVA with Fisher's least significance post-hoc analysis as appropriate for the data set. $P \leq 0.05$ was considered significant.

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