Research Paper

Paternal Insulin-like Growth Factor 2 (Igf2) Regulates Stem Cell Activity During Adulthood

Vilma Barroca, Daniel Lewandowski, Agnieszka Jaracz-Ros, Sylvie-Nathalie Hardouin *

INSERM UMR 967, 92265 Fontenay-aux-roses cedex, France
CEA/DSV/iRCM, 92265 Fontenay-aux-roses cedex, France
Université Paris-Diderot, Paris 7, 92265 Fontenay-aux-roses cedex, France
Université Paris-Sud, Paris 11, 92265 Fontenay-aux-roses cedex, France

ABSTRACT

Insulin-like Growth Factor 2 (IGF2) belongs to the IGF/Insulin pathway, a highly conserved evolutionarily network that regulates growth, aging and lifespan. Igf2 is highly expressed in the embryo and in cancer cells. During mouse development, Igf2 is expressed in all sites where hematopoietic stem cells (HSC) successively migrate, then its expression drops at weaning and becomes undetectable when adult HSC have reached their niches in bones and start to self-renew. In the present study, we aim to discover the role of Igf2 during adulthood. We show that Igf2 is specifically expressed in adult HSC and we analyze HSC from adult mice deficient in Igf2 transcripts. We demonstrate that Igf2 deficiency avoids the age-related attrition of the HSC pool and that Igf2 is necessary for tissue homeostasis and regeneration. Our study reveals that the expression level of Igf2 is critical to maintain the balance between stem cell self-renewal and differentiation, presumably by regulating the interaction between HSC and their niche. Our data have major clinical interest for transplantation: understanding the changes in adult stem cells and their environments will improve the efficacy of regenerative medicine and impact health- and life-span.

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1. Introduction

IGF2 is a member of the IGF/Insulin signaling (IIS) pathway, an evolutionarily conserved network that also comprises IGF1 and Insulin, which regulates cell proliferation, differentiation, survival and longevity (Germs and Partridge, 2001; Kenyon, 2010; LeRoith and Yakar, 2007). In human beings Igf2 is widely expressed lifelong and it is involved in growth (Ekström et al., 1995; Begemann et al., 2015). In the mouse, Igf2 is ubiquitously and abundantly expressed during development, but its expression stops at weaning (Baker et al., 1993; DeChiara et al., 1991). Igf2 regulates the development of fetal and adult cortical neural stem cells (Ferrón et al., 2015; Lehtinen et al., 2011). It is also highly expressed in all sites where hematopoietic stem cells (HSC) successively migrate and expand during development (Alvarez-Silva et al., 2003; Mascarenhas et al., 2009; Zhang and Lodish, 2004), but becomes undetectable when HSC reside in the bones of weanlings. The function of IGF2 in adulthood is unclear. In adult mice, Igf2 appears to be re-expressed in specific cell types during regeneration (e.g. Alzhavanov et al., 2010; Hovey et al., 2003; Zhou et al., 2012). As tissue development, homeostasis and response to injuries are ensured by stem cells that are present in the different tissues, these data suggest that Igf2 is involved in organ maintenance, and raise the question of its role in the biology of adult stem cells.

As a potent mitogen, IGF2 has been shown in vivo to promote regeneration of tissue mass by increasing cells numbers, and in vitro to expand fetal and adult stem cell populations (Zhang and Lodish, 2004). An increase in IGF2 can lead to organ overgrowth (Ping et al., 1989) or participate in the rapid conversion of primary cells to malignancy (Cui, 2007; Hernandez et al., 2003; Randhawa et al., 1998), whereas a decrease in IGF2 reduces embryo cell number (Rappolee et al., 1992) and results in dwarfism (Gicquel et al., 2005). Igf2 expression is controlled through genomic imprinting, a unique epigenetic regulation that causes genes to be expressed according to their parental origin. This results in activation of the paternally inherited Igf2 allele and repression of the maternal Igf2 allele (Ferguson-Smith, 2011).

Systematic gene profiling has recently revealed a predominant expression of imprinted genes in somatic stem cells (Berg et al., 2011). Imprinted genes were shown to support self-renewal of neural and lung stem cells (Ferron et al., 2015; Zacharek et al., 2011), to restrict HSC proliferation (Kubota et al., 2009), to inhibit the Pi3K-mTOR pathway to preserve HSC function (Qian et al., 2016) and HSC quiescence (Venkatraman et al., 2013).

The Igf2 gene is under the control of four alternative promoters (P0–P3) (Supplementary Fig. 1a). The P2 promoter drives the Igf2P2 transcripts, the most broadly and abundantly expressed Igf2 transcripts,
that are direct targets for the pluripotency factor lin28 and for IGF2bp (IMP). While IGF2 is mono-allelically expressed in differentiated cells that circulate in the blood, it is bi-allelically expressed from its P2 promoter in immature cells from adult bone marrow (Morrison et al., 2000), a feature that is usually strongly associated with active proliferation of normal or leukemic cells (Vorwerk et al., 2003).

The tight regulation of its dosage and allelic expression led us to hypothesize that IGF2 has a very precise function in stem cells: early in development, IGF2 would ensure the adequate expansion of stem cells present in developing organs, then during post-natal life, IGF2 would contribute to stem cell homeostasis. We proposed that the IGF2 locus or its paternal allelic expression might be a hallmark of tissue stem cells and that the IGF2P2 transcripts are involved in the biology of HSC, so that removing the IGF2P2 transcription unit in mice would disturb stem cells. We previously created IGF2P2-deficient mice and demonstrated their deficit in Mesenchymal Stem Cells (Hardouin et al., 2011).

In the present study we investigate the function of IGF2 in adult HSC. Hematopoiesis is a hierarchy where the long-term repopulating-HSC (LT-HSC) that reside in the bone marrow continuously regenerate blood cells for the life of the animal. HSC have two unique properties: self-renewal that avoids depletion of the LT-HSC pool, and multipotent differentiation that gives rise to transiently constituting ST-HSC (Short-Term HSC) then to MPP (uncommitted Multipotential Progenitors) and eventually to mature cells (Supplementary Fig. 1b). ST-HSC and MPP are fast cycling cells for increasing cell mass and further differentiation to specific cell lineages (Yang et al., 2005). Homeostasis requires a balance between LT-HSC quiescence and activity to prevent bone marrow from exhaustion or overgrowth (Li and Clevers, 2010). Discovering the factors underlying HSC regulation is of great importance to both basic biology and the development of clinical applications of HSC.

In this study, we observed IGF2 preferential expression in Long-Term-HSC and we studied lifelong consequences of IGF2 deficiency on adult HSC. We were able to show how this potent mitogen and survival factor regulates the pool of HSC in steady-state conditions and during organ regeneration. We discovered that the HSC pool is subjected to an age-related decline that occurs at mid-life and that lifelong deprivation of IGF2 avoids this age-related decline. Our data showed that diminished IGF2 levels during adulthood appear beneficial for HSC in the long-term, in coherence with the protective role of the IGF1/Insulin pathway toward the organism. Importantly, we show that down-regulation of IGF2 constitutes a promising way to increase stem cells production while decreasing mobilization, avoiding the evolution to a cancerous state. This can have major clinical interest for transplantation, especially in elderly.

2. Material and Methods

2.1. Mice

Mice deficient for IGF2P2 were generated and maintained in a 129S background. As the phenotype of the IGF2 mutation is paternally transmitted, offsprings from heterozygous fathers and wild type (wt) or heterozygous mothers were used in this study and genotyped as reported (Hardouin et al., 2011). Control mice were either the littermates with no mutation, or 129S mice ubiquitously expressing a YFP transgene (7AC5/EYFP line, gift of Dr. Nagy, Toronto). All animal procedures were carried out in accordance with French Government regulations (Services Vétérinaires de la Santé et de la Production Animale, Ministère de l’Agriculture).

2.2. Flow Cytometry Analysis and Sorting (Fluorescence Activated Cell Sorting)

Bone marrow (BM) cells were flushed from femurs, tibiae, iliac crests and humeri. Peripheral blood cells were collected from the retro-orbital vein. Spleens were crushed, cells filtered. After red blood cells lysis with 500 mM NH₄Cl solution, bone marrow and spleen mononuclear cells and blood leukocytes were either phenotyped or sorted using antibodies from Becton Dickinson.

We ensured that the usual immuno-phenotyping strategy developed for hematopoietic cells of C57BL/6j mice allowed the analysis of cells from our 129S mouse lines (Supplementary Fig. 1b & d).

2.2.1. Cell phenotyping

Hematopoietic Stem and Progenitors Cells (HSPC) were labeled using biotin-conjugated anti-mouse lineage antibodies (Miltenyi Biotec) followed by eFluor 450- streptavidin (eBioscience), APC-anti-c-Kit, PE-anti-Sca-1, PE-Cy7-anti-CD16/32 and Alexa-Fluor 700-anti-CD34. Mature cells were identified as follows: granulocytes were labeled with APC-anti-Gr1 and PE-anti-CD11b; myeloid cells were positive for CD11b and/or Gr1; lymphoid cells were identified with PECy7-anti-B220 for B lymphocytes, APC-anti-CD3 for T lymphocytes, PE-anti-CD45b and anti-PE-Cy7-anti-NKGD2 for Natural Killer (NK) cells. Cells were washed then data were acquired either on a LSRII (Becton Dickinson) or on a Cyan ADP (Beckman Coulter) and analyzed using Flowjo software (Treestar).

2.2.2. Cell sorting

Hematopoetic stem cells and uncommitted progenitors (LKS +: Lin− Kit+ Sca-1+ ) and committed progenitors (LKS −: Lin− Kit+ Sca-1− CD16/32− CD34− CD45b−) were injected into wt recipient mice. Recipient mice were analyzed 8 months later.

Sorted LKS+ cells were isolated from bone marrow of 4 months old 129S wt mice expressing a YFP (FITC+) transgene and from 129S IGF2P2 (FITC−) mice. Sorted LKS+ cells used as donor cells were injected in anesthetized mice by the retro-orbital vein in 100 μl PBS. For BM reconstitution, 5000 LKS+ cells from IGF2P2 or wt mice were injected into recipient mice; for reciprocal BMT, 2.67 × 10⁶ cells of whole wt BM (eq. 1068 LKS+ cells) per mouse were injected into wt and IGF2P2 recipients; for competitive BM reconstitution, 8000-sorted LKS+ (4000 wt and 4000 IGF2P2) were injected into recipient mice. Recipient mice were lethally irradiated 24 h before transplantation with 10 Gy (1 Gy/min) of total body irradiation from a 137Cs source. Transplanted mice were maintained under antibiotic therapy for one month with daily health checking and were analyzed 8 months later. All transplantations were made with syngeneic mice of the same age. To measure hematopoietic reconstitution in transplanted mice, peripheral blood was collected at the indicated times after transplantation, red blood cells were lysed and the presence of wt (FITC+) versus IGF2P2 (FITC−) cells was evaluated by flow cytometry. Then the presence of wt and IGF2P2 cells was measured in different cell compartments by flow cytometry as described above.

2.4. Self-renewal Analyses

Equal number of IGF2P2 and wt cells from 4 months old mice were transplanted into lethally (10.0 Gy, 1 Gy/min) irradiated wt recipients. Eight months post-transplant, the primary recipients were sacrificed and their BM cells injected into lethally irradiated secondary recipients, and such serial transplantation was continued once more. BM cells of...
the primary, secondary, and tertiary recipients were analyzed eight months post-transplant: by evaluating the proportion of wt (FITC +) and Igf2P2 (FITC −) cells, and by staining the cells as described above, therefore allowing examining the percentage of wt and Igf2P2 cells in the stem cells and progenitors compartments.

2.5. Cell Proliferation and Apoptosis Analyses

Bone marrow mononuclear cells were depleted of lineage-committed cells using the Cell lineage depletion kit (Miltenyi Biotec). Lin − cells were stained with the cell surface markers APC-anti-c-Kit, PE-Cy7-anti-Sca-1, PerCP-Cy5.5-anti-CD16/32 and FITC-anti-CD34 antibodies. For cell proliferation analysis, stained cells were fixed in paraformaldehyde 1%, permeabilized with 0.1% Triton X-100 and stained with PE-anti-Ki-67 (BD Pharmingen) and with Hoechst. For analysis of apoptotic cells, stained cells were then labeled with PE-anti-Annexin V according to the manufacturer’s instruction (Biotium) and with Hoechst. Apoptotic cells were defined as Annexin V + Hoechst − cells. Cells were analyzed by flow cytometry on a LSRII flow cytometer (BD) using FlowJo software (Treestar).

2.6. Stress-induced Mobilization of Hematopoietic Stem and Progenitor Cells (HSPC)

Granulocyte colony-stimulating factor (G-CSF) was administered subcutaneously twice a day for 2 days and the morning of the third day at the dose of 2.5 μg per mouse. AMD3100 was injected subcutaneously in one dose of 5 mg/kg per mouse. Blood samples were collected 3 h after the last injection of G-CSF or 1 h after AMD3100 injection. Blood leukocytes were counted with an automatic hematology analyzer 3 h after the last injection of G-CSF or 1 h after AMD3100 injection.

2.7. Colony-forming Unit Assay

The colony-forming unit (CFU) assay (Method Cult 3434, StemCell Technologies, Canada) was performed according to the manufacturer’s instructions. Fifty microliters of NH4Cl lysed blood from mice treated with G-CSF or AMD3100 were added to 2 ml of complete Methocult and seeded in 35 mm culture dishes. Culture dishes were transferred into an incubator at 37 °C, in a 5% CO2 atmosphere with 95% humidity. After 7 days cultures were evaluated for the presence of multi-potential CFU i.e. CFU with multi-lineage progenitors (CFU-GEMM: granulocyte-erythrocyte-monocyte-megakaryocyte), of bi-potential CFU-GM (CFU-granulocyte-macrophage), of CFU-G (CFU-granulocyte), CFU-M (CFU-macrophage) and CFU-E (CFU-erythroid).

2.8. Histology

Bones and spleens were dissected, fixed in formalin. Samples were then dehydrated, embedded in wax, sectioned then stained with eosin-hematoxylin or Masson trichrome.

2.9. Quantitative RT-PCR

RNA was prepared from flow-sorted HSC and progenitors using the RNeasy Plus Micro kit from QIAGEN, then reverse transcribed using the SuperScript II Reverse Transcriptase kit from Invitrogen. The cDNA was used as template for real-time PCR using Power SYBR Green Master Mix (Applied Biosystem) in Eppendorf detection system. Primers used in Figs. 2b, 6c, d, were obtained from Eurogentec and are listed in Supplementary data, Supplementary Table 1, primers used in Fig. 6a, b are similar to those used in Kusy S. et al., Cell Stem Cell 2011. The mRNA levels for genes of interest were normalized to beta actin levels present in the same samples.

2.10. Data Analysis and Statistics

All results are presented as means ± standard errors of the mean. Statistical analyses were performed using Student’s t-test. A p value <0.05 was considered statistically significant.

3. Results

3.1. Paternal Igf2P2 Deficiency Avoids Mid-life Attrition of the HSC Pool

We previously reported that deleting the main paternal Igf2 transcription unit in mice, results in Igf2 deficiency and growth retardation (Hardoun et al., 2011). To determine the onset of adulthood in wild type (wt) and in Igf2P2 mice, we compared their post-natal growths. In wt mice, the period corresponding to intense cell proliferation linking hematopoiesis and bone formation ended between 2 and 3 months of life. At 2 and 3 months of age Igf2P2 mice appeared younger than their wt littermates: their body growth was delayed (Fig. 1a) and epiphysis closure was unachieved (Fig. 1b). We used mice older than four months as adult mice. We analyzed Igf2 expression in bone marrow cells from adult mice following the cellular hierarchy presented in Supplementary Fig. 1b. In wt mice Igf2 mRNA was expressed in LT-HSC, strongly decreased in ST-HSC & MPP and committed progenitors, then undetectable upon differentiation (Figs. 1c, & 2), whereas in Igf2-deficient mice, Igf2 mRNA expression was strongly reduced and Igf2 transcripts were undetectable (Figs. 1c, 3 & 4).

We investigated the consequences of Igf2 deficiency on steady-state hematopoiesis (Supplementary Fig. 1b). Flow cytometric analysis of bone marrow cells from adult Igf2-deficient mice revealed that all blood cell types were present throughout life and that there was no apparent lineage bias: the loss of Igf2P2 did not hamper the contribution of HSC to either the myeloid (granulocytes, macrophages) or lymphoid (B, T, NK cells) lineages (Supplementary Fig. 1c). This indicated that Igf2 had no major effect on hematopoietic fate commitment.

To evaluate whether decreased levels of Igf2 had an effect on stem cells, we analyzed Igf2-deficient primitive compartments lifelong (Supplementary Fig. 1b, d). In wt mice, HSC frequency exhibited a bell shaped curve reaching a maximum at mid-life, whereas in Igf2-deficient mice, HSC frequency increased continuously until the end of life (Fig. 1d). LKS + cells (Lin − c-Kit+ Sca-1 + cells that contain LT-HSC, ST-HSC & MPP) behaved similarly, except in the young 2-month-old LKS + Igf2-deficient cells. Thus, low Igf2 level led to the maintenance of aging HSC, suggesting that the level of Igf2 expression had a role in regulating the number of LT-HSC in BM.

As Igf2 deficiency resulted in HSC expansion, it could also alter the amplification between HSC and progenitors or differentiated cells. Between 2 and 20 months of life, the numbers of LT-HSC and of ST-HSC & MPP respectively increased 15-fold and 2-fold in Igf2P2 mice, but only 4-fold and 1.5-fold in wt mice (Fig. 1e and Supplementary Fig. 1e). At the same time, the numbers of committed progenitors and mature cells tripled in wt mice but only doubled in Igf2P2 mice (Fig. 1e and Supplementary Fig. 1f). These opposite behaviors resulted in 3 times more stem cells and 2 times fewer progenitors in 20-month old mutant mice (Fig. 1e), compared with wt.

Thus, in aging mice Igf2 deficiency resulted in the progressive increase of the LT-HSC population but in their decreased ability to generate progenitors, leading to subsequent low BM cellularity.

Altogether these data showed that Igf2 deficiency resulted in the expansion of LT-HSC at the expense of differentiation into committed progenitors. This could be due to enhanced LT-HSC survival and/or self-renewal, or to decreased LT-HSC differentiation, or both.

3.2. Paternal Igf2 Modulates HSC Cell Cycle

IGF2 is a potent survival and mitogen factor, so alteration of survival or proliferation could provide an explanation for the accumulation of
Fig. 1. Igf2-deficiency increases the stem cells pool in aging mice. (a) Post-natal growth curves of wt and Igf2P2 mice (n = 12 per genotype at all ages, except at six weeks n = 4 of each genotype). Whole-mouse weight versus developmental time provides an adequate, although coarse, overall index of the growth process. (b) Representative histological sections of growth plates (denoted by the brackets) from 3-month-old wt and Igf2P2 mice. The columnar alignment of numerous chondrocytes in Igf2P2 bones, opposed to the irregular small groups of chondrocytes in the wt growth plate that is closing, indicates that the developmental stage of Igf2P2 mice corresponds to that of younger mice. Arrows show representative chondrocytes. Scale bar: 200 μm. (c) Relative expression of Igf2 transcripts in cells sorted from bone marrow of wt mice (1,2) and of Igf2P2 mice (3,4) (n = 8 of each genotype). (d) Frequencies of LT-HSC and LKS+ cells in wt and Igf2P2 mice during life (n = 10 per genotype and per age). (e) Numbers of LT-HSC, ST-HSC & MPP, committed progenitors and differentiated cells in BM from wt and Igf2P2 mice during life; n = 10 per genotype and per age. Data represent the mean ± SEM of at least 3 independent experiments. See also Supplementary Fig. 1.
Igf2-deficient HSC in the bone marrow. However, we found that the apoptotic rate of Igf2-deficient HSC was similar to wt, as evaluated by staining HSC with the apoptosis marker Annexin-V (not shown). We therefore examined the cell cycles of Igf2P2 and wt bone marrow cells. As the phenotype of Igf2P2 deficiency was more pronounced after mid-life, we compared the cell cycle distributions of primitive cells before (8 months) and after (20 months) mid-life. There were more LT-HSC and ST-HSC & MPP in the G0 phase of the cell cycle of 8 months old Igf2P2 mice and significantly more ST-HSC & MPP in the G0 phase in aging Igf2P2 mice than in the corresponding wt populations. There was no significant difference in cell cycle distribution of committed progenitors between Igf2P2 and wt (Fig. 2a).

We quantitated transcripts levels of cell cycle regulators by real time PCR (Supplementary data, Supplementary Table 1) on 8- and 20-month old cells (Fig. 2b). Igf2P2 cells had lower levels of cyclinD1 mRNA and higher levels of the cell cycle inhibitor p21. In Igf2-deficient HSC, the increased expression of cell cycle regulators that promote HSC quiescence (p57), that block the G0 to G1 passage (p21) and that decrease progenitor cell cycle activity (p27) was more pronounced in Igf2P2 than in wt mice.}

Fig. 2. Paternal Igf2P2 modulates HSC cell cycle. (a) Proportions of LT-HSC, ST-HSC & MPP and committed progenitors from 8- and 20-month-old wt and Igf2P2 mice (n = 8 of each genotype), in the different phases of the cell cycle. *p-value < 0.01. (b) Fold changes in expression of cell cycle regulators in Igf2P2 mice relative to wt: LT-HSC, LKS+ and committed progenitors from 8-month old mice (upper panel, n = 8 of each genotype) and 20-month old mice (lower panel, n = 16 of each genotype). The fold changes in mRNA expression are evaluated by q-PCR and are presented as a log2 scale: up- and down-regulated gene expressions are compared to wt level expression set to zero. Data are expressed as mean ± SEM of 3 independent experiments. See Supplementary data, Supplementary Table 1.

Fig. 3. Competitive bone marrow reconstitution reveals Igf2P2 HSC cell intrinsic properties. (a) Scheme of the competitive transplantation assay. (b) Chimerism in differentiated cells in blood of recipients, 2 months after bone marrow transplantation (BMT) (n = 8 of each genotype). (c) Chimerism in bone marrow stem cells, progenitors and differentiated cells from long-term recipients (n = 8 of each genotype). (d) Scheme of the serial transplantation assay. (e) Chimerism during serial bone marrow transplantation. Igf2P2 and wt cells were analyzed 8 months after they were transplanted into 4 primary, secondary or tertiary recipients. (f) Bone marrow cellularity decreases in serial wt recipients. (g) Example of absolute number of cells in secondary recipients. (h) Left and middle panels: representative histological sections of bones (Masson trichrome staining, scale bar: 200 μm) and spleens (hematoxylin and eosin staining, scale bar: 50 μm) from long-term secondary recipients. Arrows in spleens indicate megakaryocytes. Right panel: bones from tertiary recipient appear white due to the lack of BM cells. (i) Chimerism in spleens of primary recipients.

Data are expressed as mean ± SEM of 2 independent experiments. See also Supplementary Fig. 2.
3.3. Igf2 Deficiency Increases HSC Self-renewal

To characterize the functional defect of Igf2-deficient HSC we analyzed their reconstitution ability in vivo and used them in competitive transplantation assays. We transplanted equal numbers of LKS+ cells from adult wt and Igf2P2 mice into lethally irradiated recipient mice (Fig. 3a). We evaluated the repopulating capability by measuring the proportions of wt (FITC+) cells versus Igf2P2 (FITC−) cells in recipients.

Short-term (2 months) competitive repopulating capability was evaluated in peripheral blood of recipients and revealed the expected 1-to-1 engraftment ratio in all cell types (Fig. 3b). Eight months after transplantation, the initial 1-to-1 ratio of wt and Igf2P2 cells had shifted to a 1-to-8 ratio in favor of Igf2P2 HSC but only to a 1-to-2 ratio in favor of Igf2P2 progenitors and mature cells (Fig. 3c). These competitive bone marrow transplantation experiments suggested that Igf2-deficient adult HSC had a competitive advantage with more self-renewal, but had a lower capacity to generate progenitors and differentiated cells. To test rigorously whether Igf2 deficiency affects adult HSC self-renewal capacity we performed a serial transplantation assay (Lemischka et al., 1986). Bone marrow cells from the primary transplanted mice were transplanted into lethally irradiated secondary recipients and after 8 months bone marrow cells from the secondary recipients were transplanted into lethally irradiated tertiary recipients (Fig. 3d). We examined bone marrow for reconstitution at 8 months after each transplant. The higher percentage of Igf2P2 cells over wt cells in primary, secondary, and tertiary recipients (9-to-1 on average) (Fig. 3e and Supplementary Fig. 2) suggested a greater self-renewal capacity of Igf2P2 HSC relative to wt. In primary recipients the percentage of wt HSC was very low despite contributing ~30% to overall chimerism and mature cells. This might indicate that after the graft the wt HSC expanded fast and differentiated rather than self-renewed, and consequently rapidly reached exhaustion. Together with HSC behavior in steady-state conditions (Fig. 1d) this means that in wt HSC the rate of self-renewal is lower than the rate of differentiation so that the HSC population declines owing to exhaustion, whereas in Igf2-deficient HSC, the rate of self-renewal is higher than the rate of differentiation, so that the stem cell population expands.

Under steady-state conditions, differentiating progenitors are continuously generated to replace mature cells, but the balance between stem cell self-renewal and differentiation can adjust to physiological needs via interactions between stem cells and their environment. We observed that bone marrow cell numbers decreased over serial transplantations resulting in aplasia in tertiary recipients (Fig. 3f). Mutant HSC preferentially self-renewed and were 2 times less efficient in generating progeny and functional differentiated cells compared to wt (Fig. 3g). Analysis of bones from secondary and tertiary recipients revealed an osteosclerosis that increased over transplantations (Fig. 3h). Bone overgrowth narrowed the marrow cavity and caused extra-medullary hematopoiesis: stem cells and progenitor populations increased in the spleens where the chimerism was also in favor of Igf2P2 cells (Fig. 3h, i and j).

3.4. Paternal Igf2 Allows Long-term Hematopoietic Repopulation

We tested the ability of Igf2-deficient HSC to sustain long-term hematopoiesis without the support of wt cells. If mutant HSC were defective in the generation of progenitors and mature cells, then they would fail to produce enough cells upon transplantation into wt mice. To evaluate this possibility, lethally irradiated syngeneic wt mice were transplanted with the same numbers of either wt or Igf2P2 LKS+ cells (Fig. 4a). Two months after transplantation, the frequencies of blood cell populations were normal in all recipients, attesting that wt and Igf2P2-deficient cells had reconstituted hematopoiesis (not shown). However 5 months after the transplantation, i.e. after the transplanted HSC had self-renewed, mice that received Igf2-deficient LKS+ cells started to die (Fig. 4b). Eight months after transplantation, analysis of the survivors revealed a progressive aplasia of Igf2P2 HSC (Fig. 4c and d). The Igf2-deficient transplanted cells were unable to generate enough committed progenitors or mature hematopoietic cells but still accumulated HSC. Interestingly, this phenotype developed only at long term and generated mutant cells in proportions similar to those observed in mutant mice under steady-state conditions (Figs. 1d and 4e). This indicated that the phenotype of Igf2-deficient LT-HSC was cell autonomous and suggested that Igf2 is required for either HSC function or progenitor expansion, or both.

3.5. Igf2-deficient Environment Reduces the Proliferation of Wt HSC

The behavior of Igf2-deficient LT-HSC could be due to defects in HSC themselves but also to defects in the bone marrow microenvironment that provides the stem cell niches. To evaluate whether an Igf2-deficient bone marrow microenvironment could have affected HSC, progenitor expansion or hematopoiesis, we transplanted wt cells into lethally irradiated Igf2-deficient and wt recipient mice (Fig. 5a). Two months post-transplantation, hematopoiesis was similar in both recipients (Fig. 5b). Eight months after transplantation there was no apparent alteration in hematopoiesis (Fig. 5c) and the proportions between wt LT-HSC, committed progenitors and mature cells in Igf2-deficient recipients were similar to those observed in wt recipients (Fig. 5e), indicating that an Igf2-deficient environment did not affect the intrinsic behavior of wt HSC. However the number of bone marrow cells was lower in Igf2P2 recipients (Fig. 5d and e), similar to that observed in ungrafted Igf2P2 mice (Fig. 1e), presumably as an adjustment to the narrow bones in mutants.

3.6. Reduced Differentiation of Committed Progenitors and Disturbed Expression of Adhesion Molecules on Igf2-deficient HSC

The accumulation of mutant HSC suggested that their balance was shifted toward self-renewal at the expense of differentiation. This prompted us to analyze the expression levels of known hematopoietic regulators in Igf2-deficient LT-HSC and progenitors by quantitative RT-PCR (Supplementary data, Supplementary Table 1). In Igf2-deficient LT-HSC, we observed disturbed mRNA levels of genes that regulate self-renewal or restrain proliferation (Gfi1, c-myc, c-mpl, pbx1 and Pten) (Fig. 6a). The expression level of genes involved in certain signaling pathways was disturbed: the Tgfβ3 pathway (Tgfβ3, TgfβR1, TgfβR2, smad4) that normally plays a major role in maintaining HSC quiescence, the PI3K/Akt pathway that exhibited a decreased expression of PI3K (Phosphatidylinositol 3-kinase), of Akt (protein kinase B) and of Gsk3β (glycogen synthase kinase 3β) that all act on stem cell self-renewal (Huang et al., 2009; Sato et al., 2004) and an increase in mtor (mammalian target of rapamycin) that acts on lineage commitment (Towbridge et al., 2006). These data were coherent with the enhanced self-renewal of Igf2-deficient LT-HSC and indicated that mutant HSC might have altered responses to extracellular signals. In Igf2-deficient progenitors (Fig. 6b), we observed a reduced expression of Fgfr1, a protein that is associated with the differentiation of CMP toward GMP. As the expression level of genes involved in granulocyte differentiation was decreased in mutant cells (M-CSFR, G-CSFR, GM-CSFR), these results suggest a defective orientation of Igf2-deficient cells toward GMP. We found a disturbed expression of genes involved in myeloid differentiation (Gata2, Egr1, Ld1), in erythroid and megakaryocyte differentiation (Gfi-1, Eklf, Plu1, SpiB, Flt1), and in lymphoid differentiation (Il7R) (Fig. 6b).

The expression of differentiation factors toward most lineages was decreased in mutant cells, demonstrating lower differentiation ability, coherent with the lower number of bone marrow cells in mutant mice. These data indicated that Igf2-deficient HSC preferentially self-renew and did not differentiate efficiently, being able to undergo multilineage differentiation but with only a limited expansion. This suggested that
the defect in vivo was indirect and potentially due to a misregulated interaction with the local niche microenvironment. As the stem cell niche anchors stem cells and controls their self-renewal and progeny production, perhaps mutant HSC had modified the expression of adhesion molecules, so that they were retained in the differentiation inhibiting microenvironment of the niche (Zhang et al., 2003). We analyzed the expression of molecules known to play a role in adhesion and trafficking (Fig. 6c and d; Supplementary data, Supplementary Table 1).

Fig. 4. Paternal Igf2P2 allows long-term hematopoiesis. (a) Scheme of the transplantation assay. (b) Survival curve of recipients after bone marrow transplantation (BMT). (c) Bone marrow cellularity in long-term survivors. (d) Bone marrow cellularity in ungrafted mice and in survivors. Bone marrow cellularity decreases in long-term survivors (n = 8 per genotype). (e) Absolute numbers of cells in long-term survivors (n = 8 per genotype). Data are expressed as mean ± SEM of 2 independent experiments.

Fig. 5. Igf2P2 deficient HSC have an extrinsic defect. (a) Scheme of the transplantation assay. (b) Short-term hematopoiesis in blood, 2 months after bone marrow transplantation (BMT) (n = 8 per group). (c) Long-term hematopoiesis in bone marrow, 8 months after BMT (n = 8 per group). (d) Bone marrow cellularity in ungrafted mice and in mice 5 months after transplantation. Bone marrow cellularity decreases in long-term recipients (n = 8 per group). (e) Absolute numbers of cells in recipients (n = 8 per group). Data are expressed as mean ± SEM of 2 independent experiments.
Igf2P2 LT-HSC expressed low levels of CXCR4 and mmp9 but higher levels of CD44. The opposite was observed for committed progenitors, which also expressed high levels of Cdc42 that is involved in HSC adhesion, polarity and aging (Florian et al., 2012). Furthermore, the HSC from Igf2P2 mice expressed disturbed levels of CXCR4, the receptor of SDF-1, that are both important mediators of HSC homing. These results were consistent with the hypothesis that the mutants fail to differentiate due to their inability to detach from the differentiation preventive niche. Collectively, our data indicate that Igf2 controls the balance between stem cell self-renewal and differentiation, presumably by regulating the interaction between HSC and their niche.

3.7. Paternal Igf2 Regulates Egress of HSC

Our data indicate that Igf2-deficient HSC may have a deregulated interaction with their bone marrow stem cell niche. Indeed, hematopoietic cells have the ability to migrate in and out of the bone marrow, during homeostasis and stress conditions (Lapid et al., 2008–2012; Vermeulen et al., 1998) and deficient bone resorption has been proposed to contribute to the release of HSC from bone marrow during mobilization (Kollet et al., 2006). The osteosclerosis observed in long-term recipients could result from defective bone resorption and alter HSC mobilization. We therefore evaluated...
**Igf2P2** primitive cells for alterations in their release into peripheral blood.

We found fewer cells circulating in the blood of adult *Igf2P2* mice compared to wt in steady-state conditions (Fig. 7a). We then addressed the capacity of *Igf2P2* primitive cells to be mobilized to peripheral blood using mobilizing drugs: G-CSF that loosens the adhesion between stromal cells and HSPC via the activation of a cascade of proteases liberated from leukocytes (Roberts et al., 1997) and AMD3100 that specifically cleaves the SDF-1/CXCR4 interaction to separate hematopoietic cells from stromal cells (Broxmeyer et al., 2005). G-CSF or AMD stimulation resulted in similar fold increase in leukocytes recruited from wt and *Igf2P2* mice (Fig. 7b). After G-CSF treatment the number of progenitors increased 5-fold in wt and only 3-fold in *Igf2P2* mice. AMD3100 mobilization increased LKS+ 6-fold in wt and only 4-fold in *Igf2P2* mice (Fig. 7b and Supplementary Fig. 3). This weak mobilization suggested that, compared to wt, *Igf2P2* cells had much stronger interactions with the bone marrow microenvironment. This would explain the expansion of *Igf2P2* primitive cells at the expense of wt cells during competitive bone marrow transplantation.

Next we examined in vitro the clonogenic potential of primitive cells mobilized to the peripheral blood. We cultured them in semi-solid methylcellulose then qualitatively and quantitatively scored colony-forming units (CFU) (Fig. 7c). These clonal assays did not show differences in lineage composition when grown in erythroid-myeloid-promoting conditions, as all classes of erythroid and myeloid progenitor cells were present. The 1-hour AMD3100 treatment mobilized similar proportions of wt and *Igf2P2* cells, but the proportion of *Igf2P2* multipotent colonies CFU-GEMM was lower compared to wt (Fig. 7c). The 3-days treatment with G-CSF mobilized lower proportions of *Igf2P2* compared to wt, and the proportions of *Igf2P2* CFU-GEMM were decreased compared to wt (Fig. 7c).

Collectively, these data suggested that *Igf2P2* deficiency decreased the mobilization of stem cells and progenitors and modified the interactions with the BM microenvironment. This likely resulted in the stronger anchoring of *Igf2P2* cells to the BM stroma, which hampered wt cells function during competitive bone marrow transplantation. These data suggest that *Igf2* expression levels might be crucial for the control of the interactions between HSC and niche cells.

**4. Discussion**

The contribution of *Igf2* to adult homeostasis is poorly understood. Using a long-term in vivo study, we were able to show that *Igf2* is a potent regulator of adult hematopoietic stem cells and hematopoiesis throughout life. We observed *Igf2*-specific expression in adult stem cells and we discovered that mid-life is a critical point for HSC: the
pool of HSC is well preserved until mid-life but decreases thereafter. Igf2-deficient mutants are able to avoid this age-related attrition of the stem cell compartment, by continuously increasing the number of HSC, promoting their self-renewal while reducing their differentiation and decreasing their mobilization. These opposite behaviors of wild type and Igf2-deficient HSC lead to opposite consequences in steady-state conditions: wild type HSC are fast proliferating cells that result in HSC expansion in the short term but exhaustion in the long-term, while Igf2 deficiency unexpectedly proves beneficial to HSC as it confers a sustained self-renewal resulting in a strong advantage over wild type cells during competitive repopulation.

Thus, lowering Igf2 levels during adulthood appears to be beneficial in the long-term to maintain tissue stem cell homeostasis and regeneration with age, a counterintuitive and unexpected conclusion. One would expect that Igf2 favors HSC proliferation whereas Igf2 deficiency favors HSC quiescence. However, our experimental data demonstrate that lowering Igf2 level results at mid-life in expansion of the HSC pool and better repopulation capacity but limited differentiation. Recently a link between the IGF pathway and HSC quiescence has been revealed: modification of the maternal imprint at the Igf2-H19 locus resulting in Igf2 overexpression triggered HSC proliferation that ended in HSC exhaustion (Venkatraman et al., 2013). Inversely, Igfr−/− mutants exhibit reduced Igf2 levels, an increase in LT-HSC pool and better repopulation capacity. All these data suggest that low levels of Igf2 are more appropriate for the maintenance of the stem cell pool. Reduced Igf2 level at the onset of aging would be an age-dependent adjustment to maintain hematopoiesis, whereas high levels of Igf2 would result in excessive proliferation of HSC and differentiation into progeny, leading to HSC exhaustion. High levels of Igf2 would thus be necessary and adequate for intense proliferation during a limited period of time, as HSC expansion during intra-uterine development (Alvarez-Silva et al., 2003). High Igf2 is also necessary for the rapid expansion of fetal and adult HSC in vitro (Zhang and Lodish, 2004), for the maintenance of self-renewal of mammalian pluripotent stem cells (Bendall et al., 2007) and of most stem cells that are cultured in media where pharmacological concentrations of Insulin target all Igf2 receptors (i.e. Igf-R, hybrid Ins/IGF-R and Insulin-R) (Wang et al., 2007).

Our findings fit with data obtained in mice and humans showing a progressive decline of the other growth hormones (GH and IGF1) levels during aging (Bartke et al., 2013). This suggests that starting at mid-life, decreasing Igf2 expression would be optimal for long-term tissue stem cells maintenance. Growth signaling pathways seem to control lifelong adjustment of stem cells self-renewal and differentiation. Among them, the PI3K/Akt/VS3/mTOR pathway is an important mediator of Igf2 action, with PI3K and Akt having well-established roles in promoting the maintenance of stem cells (Storm et al., 2007; Watanabe et al., 2006). Gsk3 plays an essential role in regulating the balance between HSC self-renewal and lineage commitment (Huang et al., 2009). Reduced Gsk3 may directly expand HSC while activating mTOR that acts on lineage commitment (Towbridge et al., 2006). It is known that activation of the IGF pathway is markedly activated in wild type 129S mice but is constitutively low in wild type C57BL/6 mice (Xu et al., 2014). We discovered that HSC frequency in wild type 129S mice is similar to that of the fast cycling HSC from wild type DBA2, whereas HSC frequency in Igf2-deficient 129S mice is similar to that of the slow-cycling HSC from wild type C57BL/6 mice (de Haan et al., 1997 & de Haan and Van Zant, 1999). This suggests that activation of the IGF pathway might determine the frequency of the HSC population. These studies demonstrate that the precise level of Igf2 that is essential to maintain HSC homeostasis is finely tuned through imprinting that adjusts adult stem cell activity and quiescence. Igf2 is close to H19 in a cluster of imprinted genes. H19 decreases Igf1r and inhibits proliferation. The H19 differentially methylated region (H19-DMR) controls the reciprocal expression of paternal Igf2 and maternal H19 (Bartolomei et al., 1991; DeChiara et al., 1991). The experimental deletion of the maternal H19-DMR in HSC lowers H19 expression, increases Igf2 expression and leads to HSC proliferation (Venkatraman et al., 2013), demonstrating that maternal imprinting maintains adult HSC pool and quiescence via low Igf2 expression. The data we obtained with Igf2-deficient mice are coherent with these results and suggest that paternal Igf2 maintains adult HSC activity. HSC are also under the tight control of p57 (Mascarenhas et al., 2009) that is oppositely imprinted to Igf2 in the same gene cluster and has opposite effects on the cell cycle: Igf2 promotes the G1/S transition through the cell cycle whereas p57 is necessary for HSC quiescence and self-renewal (Caspari et al., 1999; Matsumoto et al., 2011). We show that Igf2 deficiency, which increases the HSC pool and results in better repopulation capacity, also increases p57 presumably to favor HSC self-renewal and maintain hematopoiesis. Conversely, Igf2 overexpression would boost HSC progression into the cell cycle, leading to proliferation with a risk of exhaustion of the HSC pool on the long term. The data of Thomas et al. 2016 show that in this situation p57 increases and limits the progression of HSC through the cell cycle, preserving the HSC pool and a better repopulation capacity. Our two studies suggest that p57 would counterbalance the effects of Igf2 on the HSC pool in order to protect it.

Loss of Igf2 also results in reduced HSC differentiation, presumably by retention of HSC in the BM niche due to disturbed expression of adhesion molecules. In our mouse model, Igf2 deficiency starts in utero and occurs in HSC and in their environment, including stromal cells and bone trabeculae that provide the stem cells niches. Igf2-deficient mice exhibit impaired bone resorption and dense networks of trabeculae. These defects presumably contribute to the poor mobilization of adult Igf2P2 cells. However, some defects also develop during adulthood, for example osteoporosis is only observed in long-term recipients of serial transplantations. The environment regulates HSC adhesion and egress, two critical steps that help HSC maintain a balance between self-renewal and lineage commitment in order to maintain homeostasis. Although reciprocal transplantation demonstrated that an Igf2-deficient BM microenvironment does not seem sufficient to cause the Igf2-deficient HSC phenotype (Fig. 5), our data show that Igf2 regulates interactions between HSC and their stem cell niche and may influence the balance between self-renewal and differentiation. This is more critical during aging, where wild type HSC cycle and differentiate until exhaustion of the pool, while Igf2 deficiency protects hematopoiesis from age-related decline, increases HSC retention in their niches and favors HSC self-renewal versus differentiation into progenitors over age. These data suggest that Igf2 deficiency might be essential for HSC maintenance and lead to the hypotheses that low IGF supports healthy tissue aging while continuously high IGF signals would be deleterious for tissue regeneration. When considering the IGF/Insulin longevity pathway, which promotes life and health extension through decreased activity, reducing or slowing down Igf2 level would represent a means for stem cells to survive when faced with cellular stressors, resulting in increased cellular health and health span.

We propose that paternal Igf2 levels regulate the intrinsic requirement of stem cells from the zygote stage. Widespread Igf2 expression during embryonic development would contribute to the rapid and harmonious development of organs, then its restriction to adult stem cells would control body homeostasis lifelong, by regulating temporal changes in stem cells (see Graphical Abstract). The maintenance of broad Igf2 expression in adult humans but not in adult mice must reflect their different needs in cell proliferation and regeneration, related to different sizes and life spans. Our study adds imprinting and heterochrony as new dimensions to the IGF/Insulin pathway, and reveals that the evolutionary conservation of Igf2 parental imprinting might play a key role in longevity by protecting tissues. Altogether, these data suggest that Igf2 might act throughout life as regulator of a stem cells intrinsic program, coherent with the protective role of the IGF/Insulin pathway toward the organism.

In conclusion, we have uncovered a role for Igf2 that is linked to health-span rather than to lifespan. We show that lowering Igf2 levels is a promising way to enhance the HSC pool, while avoiding the
uncontrolled proliferation and migration of immature cells that would lead to transformation events often associated with aging (Sun et al., 2014). Our study helps to understand changes occurring in stem cells and their environment during aging and tissue regeneration (Vooj and Jones, 2010). This has major clinical interest for transplantation in elderly people: understanding the changes in aging stem cells and their environments will improve the efficacy of regenerative medicine and extend health- and life-span.

Supplementary data can be found online at http://dx.doi.org/10.1016/j.ebiom.2016.11.035.

Conflict of Interest

None of the authors have conflict of interest.

Author Contributions

VB performed animal work and histology; DL and AJR performed animal work and FACS experiments; SNH conceived the project, performed and interpreted the experiments, wrote the paper.

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