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Reduced number of VSELs in bone marrow of Growth Hormone transgenic mice indicates that chronically elevated Igf-1 level accelerates age-dependent exhaustion of pluripotent stem cell pool – novel view on aging

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Senescence is an inevitable consequence of life. As a result of exposure to intrinsic- as well as extrinsic-aging factors, cellular aging is triggered in stem cells (SCs) by gradually accumulating DNA damage due to action of reactive oxygen species (ROS) and epigenetic changes.[1] Thus, aging can be envisioned, at the cellular level, as a result of altered cell function in response to changes in DNA structure that directly affects proper gene expression. In addition, SCs also age by intrinsic mechanism encoded by phenomenon of telomere shortening.[2]

Several well-known risk factors, such as obesity, diabetes, and lack of physical activity that lead to atherosclerosis of the cardio-vascular system and impair the function of vital organs (*e,g.*, heart, kidney, or brain), limit overall life span. It is obvious that all these risk factors somehow must ultimately have an impact on the basic units of tissue rejuvenation, which are SCs.[1] They can directly affect SCs or damage the niches in which these cells reside and thereby impair SC self renewal and differentiation. On the other hand, these risk factors may also lead to enhanced turn-over/proliferation of SCs, resulting in premature exhaustion of these cells. The elucidation of these precise mechanisms will help to develop more efficient anti-aging strategies.

Evidence accumulates that increase in calorie uptake and insulin, insulin-like growth factor (Ins/Igf) level in peripheral blood (PB) accelerates aging.[3–8] On the other hand calorie restriction and decrease in Ins/Igf signaling increases lifespan in worms, flies, and mammals. Furthermore, Insulin-like growth factor-1 (*Igf1*), Insulin-like growth factor-2 (*Igf2*) and Insulin (*Ins*) are described as stimulators of proliferation of normal and malignant hematopoietic stem progenitor cells (HSPCs).[9] While Igf1 and Igf2 signal through tyrosine kinase receptor Igf1R, Ins together with Igf2 may activate classical insulin receptor (InsR)

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Conflict Of Interest Statement
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and all factors together may activate a hybrid Igf1R/InsR. In contrast Igf2R serves as a "decoy receptor" that prevents Igf2 from binding to Igf1R or InsR. While Ins is secreted after calorie uptake from pancreatic islands, Igf1 (known also as somatomedin-C) is secreted from liver into peripheral blood in growth hormone (GH) dependent manner.[8]

Interestingly, the expression of *Igf2, Igf2R* and *Rasgrf1* (a small GTP exchange factor for Ras protein that is involved in Ins/Igf signaling) are regulated by changes in somatic imprinting. The imprinted genes play a crucial role in embryogenesis, fetal growth, the totipotential state of the zygote, and the pluripotency of developmentally early stem cells.[10] The expression of imprinted genes is regulated by DNA methylation within differential methylated regions (DMRs), which are CpG-rich *cis*-elements within their gene loci.

Recently, our group demonstrated that adult tissues including bone marrow (BM) harbor a population of pluripotent Oct4$^+$ SSEA-1$^+$Sca-1$^+$Lin$^-$CD45$^-$ very small embryonic-like stem cells (VSELs), [11] and postulated that these pluripotent stem cells (PSCs) are deposited during early embryogenesis as a backup for long term repopulating hematopoietic stem cells (LT-HSC). In fact as recently demonstrated BM-derived VSELs could be specified into hematopoietic lineage in co-cultures over OP-9 stroma cell support.[12] Furthermore, molecular analysis of VSELs revealed that their quiescence in adult BM and potentially premature depletion is controlled by epigenetic changes of some imprinted genes that regulate signaling of Ins/Igf (*e.g., Igf2-H19* locus, *Igf2R* and *Rasgrf1*). [13] Accordingly, we observed that murine BM-sorted VSELs erase the paternally methylated imprints (*e.g.*, DMRs at *Igf2-H19* and *Rasgrf1* loci); however, they hypermethylate the maternally methylated ones (*e.g.*, DMRs at *Igf2R*).[13] This epigenetic reprogramming of genomic imprinting negatively affects Ins/Igf signaling and maintains the quiescence of VSELs and protects them from premature aging and tumor formation.

Our initial studies performed on normal 4-week-old and 2-year-old mice revealed that the number of VSELs and their pluripotentiality decrease during ageing.[14, 15] To support this, VSELs from old mice show lower expression of the pluripotentiality master-regulators such as *Oct4, Nanog, Sox2, Klf4*, and *cMyc* and, at the molecular level the *Oct4* promoter in VSELs becomes hypermethylated with age and shows a closed chromatin structure. Furthermore, VSELs from old mice show the somatic type of methylation at both *Igf2-H19* and *Rasgrf1* loci, which suggests their increased sensitivity to Ins/Igf signaling.

Based on this we became interested in a role of prolonged Ins/Igf signaling in homeostasis of pool of BM-residing VSELs and VSELs-derived LT-HSC. To address better a role of Ins/Igf signaling on a pool of VSELs and HSCs we performed previously experiments in Laron dwarf mice. Due to a deficiency of GH receptor (GHR), these animals display a severe reduction in the Igf1 plasma level and do not display increase in GH-mediated Igf-1 plasma level in response to caloric uptake. Interestingly, these animals live 30–40% longer than their normal littermates.[3] Therefore, we measured the number of VSELs in BM of 20 month old murine Laron dwarfs (GHR$^{-/-}$) and corresponding normal heterozygous littermates (GHR$^{+/-}$) by FACS. Interestingly, we noticed that the number of VSELs in the BM of plasma Igf1-deficient Laron dwarfs is maintained at a 3–4-fold higher level than normal GHR$^{+/-}$ littermates during aging.[15] Our molecular analysis studies have additionally demonstrated that the *Oct4* promoter in these animals shows also a higher level of demethylation. We also observed that Laron dwarf mice have in BM i) a ~3-fold increase in the number of Sca-1$^+$c-kit$^+$lineage$^-$ (SKL) hematopoietic stem cells and ii) up to 4-fold higher number of clonogenic progenitors. [15] Of note, more recently we even found more pronounced differences in number of VSELs and HSPCs between 26 month old Laron dwarf mice and their littermates (manuscript in preparation).

To extend these studies, we employed in the current work “a reverse model” that is transgenic mice that overexpress bovine growth hormone (bGH) and thus in contrast to Laron dwarf mice display chronic elevation of Igf1 in PB. As already reported, due to increased Igf1 level these mice are living much shorter than normal littermates (up to 1 year only) and develop frequently malignancies.[16] These animals are also significantly larger than normal controls and display several symptoms of acromegaly.

In the current study 6 month old phosphoenol pyruvate carboxykinase bovine growth hormone (PEPCK-bGH) transgenic males and control normal animals were employed and we noticed that bGH transgenic mice are slightly anemic as compared to controls (Table I). The low hematocrit level (Ht) and hemoglobin (Hb) concentration (Figure 1 A) in combination with normal number of erythrocytes is indicative for a presence of microcytic anemia in these animals. Transgenic mice had also slightly elevated platelet counts (Table I). More importantly, we noticed severe reduction of Sca-1^{+}Lin^{-}CD45^{-} VSELs and Sca-1^{+}Lin^{-}CD45^{+} HSCs ratio in BM of bGH transgenic mice (Figure 1 B and C). However, at the same time we did not observe differences in number of clonogeneic progenitors (CFU-GM, CFU-Meg) except slight decrease in number of BFU-E, suggesting that defect in hematopoietic compartment was more profound at level of most primitive HSCs than hematopoietic progenitor cells (Figure 1 C), but this reduced number of HSCs still was able to maintain normal number of clonogeneic progenitors.

In parallel we performed molecular analysis of VSELs isolated from bGH transgenic and normal control mice. Figure 2 A demonstrates that Oct-4 promoter in 6 month old bGH transgenic mice-derived VSELs is hypermethylated (~72%) to the level similar as seen in HSCs. In contrast Oct-4 promoter in VSELs isolated from 6 month old normal mice still shows significant degree of hypomethylation (~30%). This suggests that VSELs in BM of bGH transgenic mice significantly lost their pluripotential character. At the same time DMR which regulates expression of H19-Igf2 locus that is hypomethylated in normal mice-derived VSELs (~1%) become hypermethylated in VSELs isolated from BM of 6 month old bGH transgenic mice. This indicates loss of imprinting and its reversal to somatic pattern and enhanced autocrine expression of Igf2 in these cells during aging (Figure 2 B). Similarly, DMR region for RasGrf1 locus, that encodes GTP-exchange factor for H-Ras involved in Ins/Igf signaling, that remains hypomethylated in VSELs from normal mice, become methylated in VSELs isolated from BM of bGH transgenic animals what again indicates restoration of somatic imprint at this locus (Figure 2 C). These epigenetic changes *in toto* lead to de-repression of imprinted genes that keep VSELs quiescent. Therefore, an enhanced Ins/Igf signaling in VSELs from bGH transgenic mice in combination with elevated plasma Igf1 level in these animals leads to premature depletion of these cells.

Overall, since the plasma Igf1 level is regulated in mice by caloric uptake and in humans by caloric uptake providing that protein uptake is also low, these data shed new light on caloric restriction, senescence, and the size of hematopoietic stem cell compartment. Based on our findings, we propose that chronically elevated levels of Igf1, resulting, for example, from high caloric uptake, may lead to premature depletion of the stem cell pool, including VSELs and HSCs, and thus be responsible for premature aging in mice.

Further studies are needed to link the effect of chronic high Igf1 signaling in VSELs with the enhanced frequency of cancer in bGH tarnsgenic mice. Since many human malignancies are activated by Igf1 and Igf2 signaling (*e.g.*, due to loss of heterozygosity or loss of imprinting at the *Igf2-H19* locus), we hypothesize that excessive activation of VSELs in an Ins/Igf-dependent manner could promote malignant transformation of these cells. [17]

Based on our previously published data Laron dwarf mice with low circulating levels of Igf1 and current observations in bGH transgenic mice with high circulating levels of Igf1, we postulate novel linkages between the Igf1 level, aging, and the stem cell compartment (Figure 3). According to our hypothesis, early in development a population of VSELs would be deposited in developing organs as a backup for tissue-committed stem cells that plays a role in rejuvenation of tissues and organ regeneration after damage. These cells seem to be protected from uncontrolled proliferation and age-related depletion by changes in imprinted genes that regulate Ins/Igf signaling. We hypothesize that the pool of VSELs residing in adult tissues including BM is regulated by the circulating Igf1 level. An increase in Igf1 level (*e.g.*, bGH transgenic animals, chronic high caloric uptake) would accelerate in an Ins/Igf-dependent manner an age-dependent depletion of the pool of VSELs and their potential to rejuvenate tissues (*e.g.,* in BM to supply LT-HSCs). By contrast, a low Igf1 level (*e.g.*, Laron dwarf mutants, caloric restriction) would have an opposite and protective effect on these cells leading to longevity.

Thus, these data further support our hypothesis that relates aging, longevity, insulin factor signaling, and high caloric uptake to the abundance and function of pluripotent VSELs deposited in adult tissues. A decrease in the number of these cells will affect pools of tissue committed stem cel (e.g., HSCs in BM) and have an impact on tissue rejuvenation and life span. Strategies to attenuate Ins/Igf stimulation of VSELs may lead to improve longevity.

Acknowledgments

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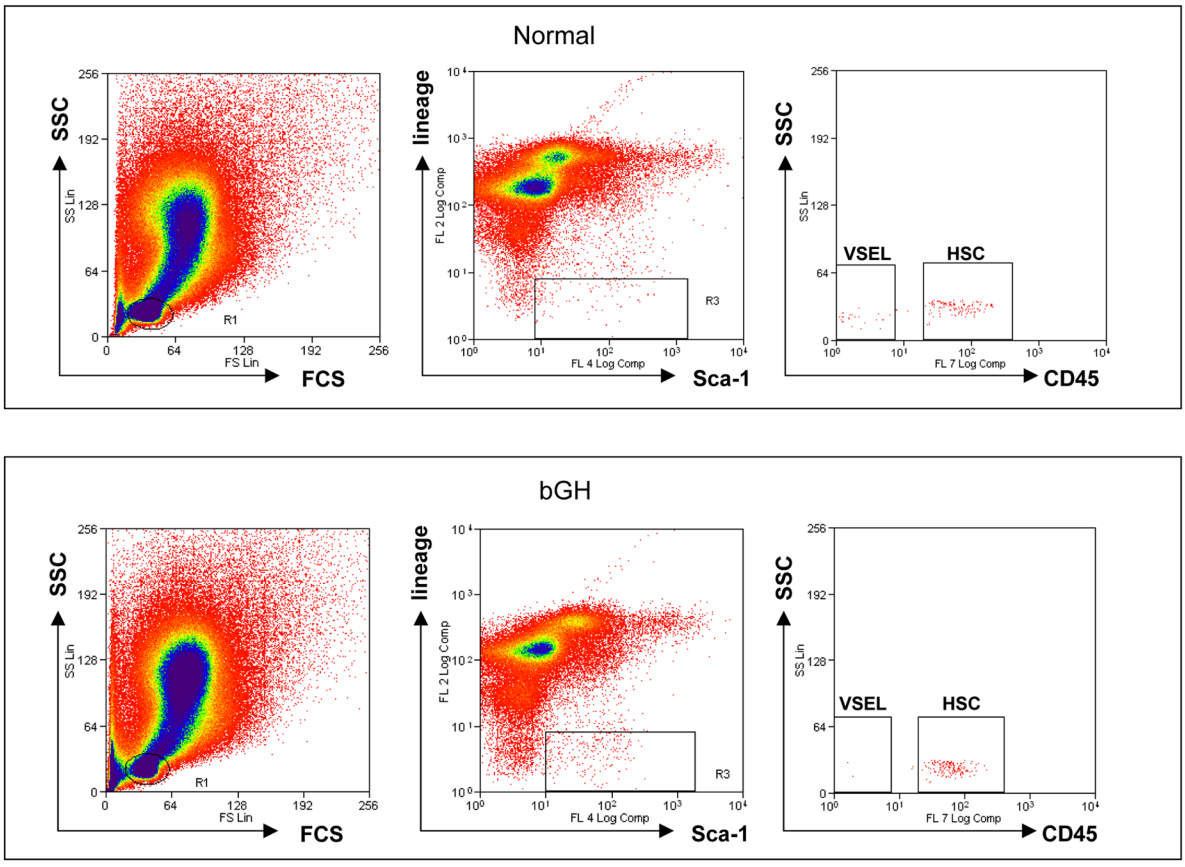

Figure 1A

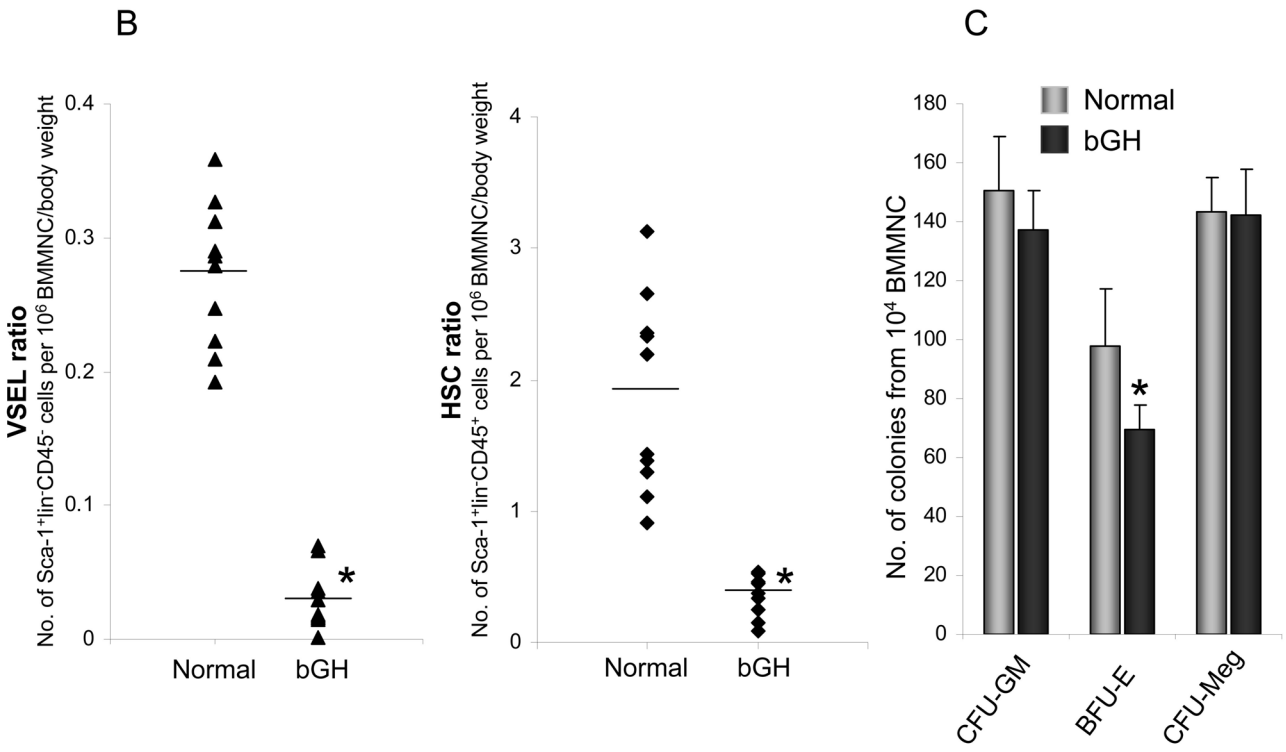

Figure 1. Increased number of VSELs and HSPCs in BM of bGH mice

(A) Sorting of Sca-1$^+$Lin$^-$CD45$^-$ (VSELs) and Sca-1$^+$Lin$^-$CD45$^+$ (HSCs) from mBM-derived MNCs by employing MoFlo High-Performance Cell Sorter. BMMNCs from region R1 are analyzed for Sca-1 and Lin expression. Sca-1$^+$Lin$^-$ population which is included in region R2 is subsequently analyzed based on CD45 antigen expression and CD45$^-$ and CD45$^+$ subpopulations visualized on dot plot, i.e., Sca-1$^+$Lin$^-$CD45$^-$ (VSELs) and Sca-1$^+$Lin$^-$CD45$^+$ (HSCs). **(B)** The ratio for Sca-1$^+$Lin$^-$CD45$^-$ (VSELs) and Sca-1$^+$Lin$^-$CD45$^+$ (HSCs) was evaluated as the number of events per 1×10^6 BMMNC/body weight by employing LSR II FACS analyzer. Analysis of Variance (ANOVA) with Bonferroni's Multiple Comparison Test *p<0.00001 as compared to normal counterparts. **(C)** The number of clonogenic BFU-E, CFU-GM and CFU-Meg from BM MNC isolated from 6 months old normal and bGH transgenic mice. Analysis of Variance (ANOVA) with Bonferroni's Multiple Comparison Test *p<0.001 as compared to normal counterparts.

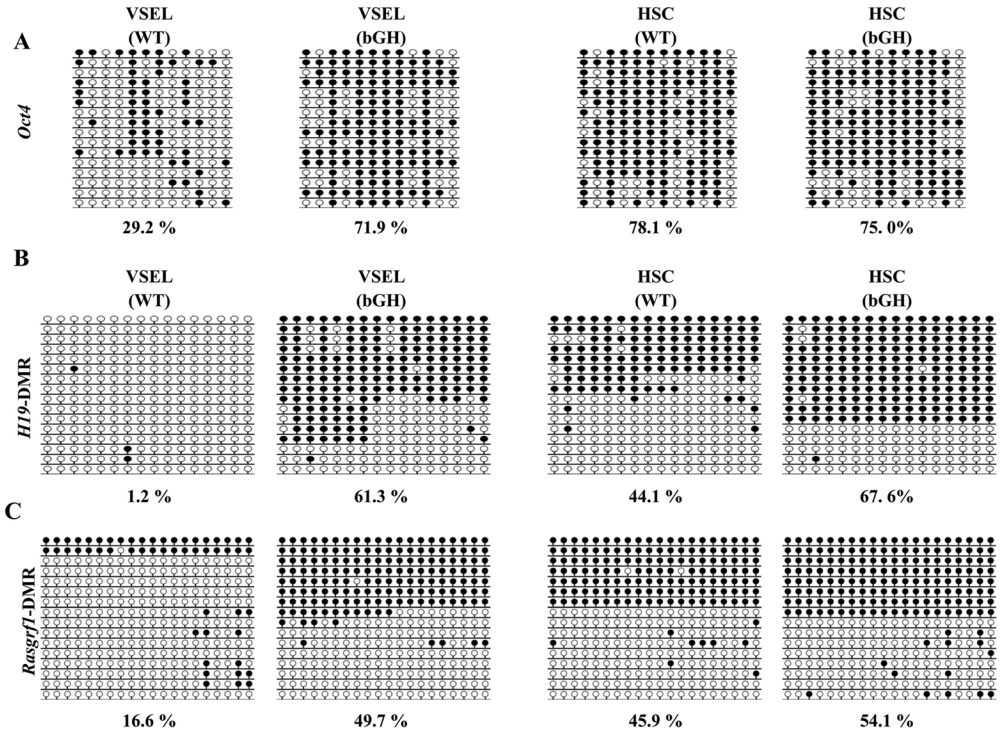

Figure 2. Change of Oct-4 promoter methylation and genomic imprinting in VSELs from 6 month old male normal and bGH transgenic mice

(A) Bisulfite-sequencing results of DNA methylation of the *Oct4* promoter in VSELs isolated from 6 month old normal and bGH transgenic mice (bGH). Methylated and unmethylated CpG sites are shown in filled and open circles, respectively. The numbers indicated below bisulfite-sequencing profiles presents the percentage of methylated CpG sites. Bisulfite sequencing results of DNA methylation of DMR for *H19-Igf2* **(B)** and Rasgrf1 **(C)** loci in VSELs isolated from 6 month old normal and b-GH transgenic mice (bGH). Methylated and unmethylated CpG sites are shown in filled and open circles, respectively. The numbers indicated below bisulfite-sequencing profiles presents the percentage of methylated CpG sites.

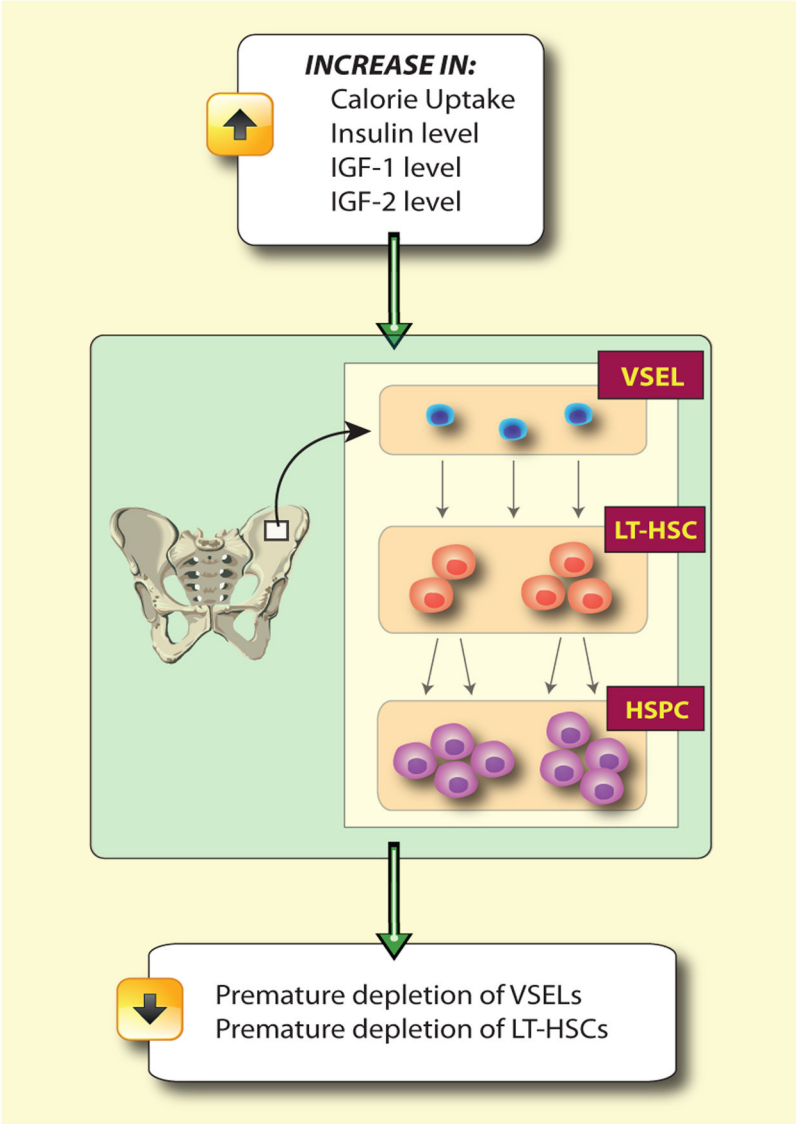

Figure 3. Hypothesis of depletion of Oct-4^{+} epiblast-derived VSELs in adult bone marrow by chronic Ins/Igf signaling in bGH transgenic mcie

Epiblast-derived VSELs are deposited in developing tissues including bone marrow as a backup population of SCs for production of tissue committed stem cells. We envision that in bone marrow VSELs are a backup population for long term repopulating hematopoietic stem cells (LT-HSCs) that give rise to hematopoietic stem/progenitor cells (HSPCs). Prolonged signaling by Insulin, Igf1, and Igf2 (*e.g.*, due to high caloric uptake or as result of GH overexpression in bGH transgenic mice) may lead to premature depletion of these cells. A decrease in the number of VSELs in bone marrow will directly affect a pool of LT-HSC and finally over time also HSPCs.

Table I

Peripheral blood parameters in male 6 month old normal and bGH-transgenic mice (n=10).

	Normal controls	bGH-transgenic mice
White Blood Cells ($10^3/\mu l$)	7.84+/−1.97	7.40+/−1.81
Neutrophils ($10^3/\mu l$)	1.95+/−0.94	1.81+/−0.54
Lymphocytes ($10^3/\mu l$)	5.18+/−2.09	4.78+/−1.26
Red Blood Cells ($10^6/\mu l$)	8.66+/−0.42	8.49+/−1.09
Platelets ($10^3/\mu l$)	1046+/−167	1369+/−198 *
Hemoglobin (g/dL)	11.53+/−0.44	9.34+/−0.99 **
Hematocrit (%)	43.70+/−1.49	35.24+/−5.15 *

* $p < 0.001$,

** $p < 0.0001$.