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Novel view of the adult stem cell compartment – a developmental story of germline and parental imprinting

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Abstract

Evidence has accumulated that postnatal tissues contain developmentally early stem cells that remain in a dormant state as well as stem cells that are more proliferative, supplying tissue-specific progenitor cells and thus playing a more active role in the turnover of adult tissues. The most primitive, dormant, postnatal tissue-derived stem cells, called very small embryonic like stem cells (VSELs), are regulated by epigenetic changes in the expression of certain parentally imprinted genes, a molecular phenomenon previously described for maintaining primordial germ cells (PGCs) in a quiescent state. Specifically, they show erasure of parental imprinting at the Igf2–H19 locus, which keeps them in a quiescent state in a similar manner as migrating PGCs. To date, the presence of these cells in adult postnatal tissues have been demonstrated by at least 25 independent laboratories. We envision that similar changes in expression of parentally imprinted genes may also play a role in the quiescence of dormant VSELs present in other non-hematopoietic tissues. Recent data indicate that VSELs expand *in vivo* and *in vitro* after reestablishment of somatic imprinting at the Igf2-H19 locus by nicotinamide treatment in response to stimulation by pituitary gonadotrophins (follicle stimulating factor, luteinizing hormone and prolactin) and gonadal androgens and estrogens. These cells could be also successfully expanded *ex vivo* in the presence of the small molecule UM177.

Keywords

Adult stem cells; primordial germ cells; imprinted genes; Igf2–H19 locus; stem cell quiescence; tissue regeneration; tumorigenesis

Introduction

Despite a vast amount of work, the hierarchy within the adult stem cell compartment is still incompletely understood. Various types of stem cells residing in postnatal tissues that possess more than one germline specification potential have been described [1–13]. The

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undisputed fact that adult tissues contain such cells gives rise to three important questions that we will discuss in this review.

The first question is related to the fact that the first stem cells specified in the developing embryo in both rodents and humans are primordial germ cells (PGCs). Therefore, one could ask: How much germline potential is present in adult stem cells? This question is highly relevant to hematopoietic stem cells (HSCs), because as we will discuss there is an intriguing developmental link between specification and migration in the embryo of PGCs and in the origin of primitive and definitive HSCs [14–17]. Moreover, these cell populations also share several markers and respond to stimulation by sex hormones (SexHs) [18–21].

The second question to be answered is: Are some of the stem cells from the embryonic stage of development deposited into and reside in adult tissues in the quiescent state? This emerging concept suggests a developmental continuum in the stem cell compartment, beginning from the fertilized zygote to adult tissue committed monopotent stem cells. If this is correct, then the end of organogenesis does not mean complete elimination of developmentally early stem cells from postnatal tissues. Such cells described as very small embryonic like stem cells (VSELs) survive in the adult body as a potential backup for tissue-committed stem cells and play a role in their turnover [22–24].

The third question is: Why do some developmentally early VSELs that express markers of pluripotency remain in a quiescent state in adult tissues, and why do they not form teratomas or complete blastocyst development? To address this question, we demonstrated that the most primitive developmentally early VSELs in adult tissues could be kept in a quiescent state, similarly as migrating PGCs, by changes in expression of certain parentally imprinted genes [25]. Proper expression of these genes is crucial for the initiation of embryogenesis and cell proliferation [26]. By contrast, these genes are expressed in embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), which enables these cells to complete blastocyst development and grow teratomas in in vivo models [25, 27].

Based on the aforementioned, we will discuss these three issues and present evidence that developmentally early VSELs, sharing several markers with PGCs and the epiblast, are kept in a quiescent state in adult tissues by changes in expression of parentally imprinted genes. In particular, we will focus on bone marrow (BM)-residing VSELs. Evidence accumulated suggests that BM-residing VSELs can be specified into HSCs, endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs).

How much germline potential is present in adult bone marrow stem cells?

The first stem cells that become specified in the earliest stages of embryogenesis in the epiblast of the post-implantation blastocyst are PGCs, as mentioned above [28, 29]. The epiblast is a precursor of the entire embryo proper, and PGCs are precursors of gametes that pass genetic information, encoded in parental DNA, and mitochondria to the next generation. These cells, endowed with developmental totipotency, become specified in the proximal part of the epiblast and, after specification, leave the embryo proper and migrate for a short period of time to the extra-embryonic mesoderm, where they begin to amplify,

make a turn, and re-enter the embryo proper through the primitive streak. While continuing to amplify in number, the PGCs migrate toward the genital ridges [30], where they settle and initiate gametogenesis. On their migratory route through the embryo proper toward the genital ridges, they cross the part of the embryo called the aorta–gonado–mesonephros (AGM) region [31].

As shown in Figure 1, the developmental route of PGCs overlaps with the emergence of the first primitive HSCs in time and space—first in the so-called blood islands at the bottom of the yolk sac and later with the emergence of definitive HSCs in the AGM region of the developing embryo proper. Both PGCs and HSCs are highly migratory stem cells and it is very likely that some of the PGCs, while migrating in the extra-embryonic mesoderm, give rise to hemangioblasts, which are precursors for both primitive HSCs and EPCs. Subsequently, while they migrate in the embryo proper through the AGM region towards the genital ridges, some of them become specified into definitive HSCs detectable in the hemangiogenic endothelium of the dorsal aorta [31–34].

Based on this close developmental overlap between PGCs and HSCs, one can ask how much germline potential is in HSCs, and, vice versa, whether germline-derived cells share genes involved in the development of both lineages. In fact, mounting evidence has accumulated that HSCs are responsive to several pituitary gonadotrophins and gonadal sex hormones (SexHs) and share certain molecular markers typical of germ development, such as the Sall4 transcription factor [35, 36]. On the other hand, germline-derived cells express the erythropoietin receptor, which is well known to be expressed by hemangioblasts and cells from the erythroid lineage. Accordingly, we demonstrated that human and murine germline-derived teratocarcinoma cells lines as well as ovarian cancer cell lines express functional erythropoietin receptors and respond to erythropoietin by chemotaxis, increased adhesion, and phosphorylation of MAPKp42/44 and AKT [37].

On the other hand, to better address the potential role of SexHs in the development of HSCs, we performed a series of experiments to address the influence of follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), progesterone, androgens, and estrogens on murine hematopoiesis [21]. We found that 10-day administration of each of the SexHs evaluated in this study directly stimulated expansion of HSCs in BM, as measured by an increase in the number of these cells (~2–3x), an observation supported by enhanced bromodeoxyuridine (BrdU) incorporation into the nuclei of these cells. The percentage of $BrdU^+$ $Sca\text{-}1^+$ Lin^- $CD45^+$ HSCs, depending on the type of SexH employed, increased from ~25% to 45–60%. This stimulatory effect paralleled an increase in the number of clonogenic BM progenitors (~2–3x). Notably, we also observed that murine $Sca\text{-}1^+$ Lin^- $CD45^+$ HSCs express pituitary and gonadal SexH receptors and respond to stimulation by phosphorylation of MAPKp42/44 and AKT. We also observed that administration of SexHs accelerated the recovery of peripheral blood (PB) cell counts in sub lethally irradiated mice and slightly mobilized HSCs into circulation. Finally, in direct *in vitro* clonogenic experiments on purified murine progenitor cells, we observed a stimulatory effect of SexHs on clonogenic potential if added with suboptimal doses of the colony stimulating factors: CFU-Mix, BFU-E, CFU-Meg, and CFU-GM. Thus, our data indicates that pituitary- and gonadal-secreted SexHs directly stimulate the expansion of stem cells in BM [21].

Finally, in further support of this developmental link between the germline and hematopoiesis, it is important to mention that several papers have described the sharing of chromosomal aberrations between germline tumors and leukemias or lymphomas, which suggests their common clonal origin [17, 38–40]. More direct evidence has also demonstrated that murine PGCs isolated from embryos, murine testes, and teratocarcinoma cell lines can be specified into hematopoietic stem/progenitor cells [15–17, 41, 42]. These findings all support a close developmental relationship between the germline and hematopoiesis.

Do early-development stem cells reside in adult tissues?

A decade ago, the concept of stem cell plasticity or stem cell trans-differentiation was proposed [6, 43–48]. Based on this idea, tissue-committed stem cells, such as HSCs, could change their fate and differentiate into stem cells for other lineages, for example, cardiac stem cells. This concept, however, did not stand up to critical examination and other explanations for why some degree of chimerism has been observed in various tissues after transplantation of bone marrow cells have been proposed. One of these alternative explanations involves the phenomenon of cell fusion [49–52].

By contrast, our team has from the beginning proposed that stem cell plasticity could be explained by the fact that the adult BM contains early-development stem cells, which we succeeded in isolating from adult murine BM cells that were slightly smaller than erythrocytes and that expressed pluripotency markers, such as Oct-4 and Nanog, which we called VSELs [24, 53]. Meanwhile, in the past several years, various cells endowed with multi-tissue differentiation potential have been identified by other investigators in adult murine or human BM and, depending on the methods for how they were isolated, assigned different names. The examples are spore-like stem cells [54], multipotent adult stem cells (MASCs) [1], mesenchymal stem cells (MSCs) [55], multi-lineage-differentiating stress-enduring (Muse) cells [56], multipotent adult progenitor cells (MAPCs) [4], unrestricted somatic stem cells (USSCs) [3], marrow-isolated adult multi-lineage-inducible (MIAMI) cells [2], or multipotent progenitor cells (MPCs) [1, 57]. Interestingly, in addition to the cells listed above, adult bone marrow has been also postulated to contain hemangioblasts [58], as well as cells that retain the potential to differentiate into gametes (Table 1) [59, 60].

This has created nomenclatural chaos, and probably several of these stem cells described as separate entities are in fact overlapping cell populations. We envision that, most likely, VSELs are at the top of the hierarchy of all of these various overlapping populations of stem cells that are endowed with pluri/multipotent differentiation potential (Figure 2) [61, 62]. In BM tissue, they can give rise to HSCs, MSCs and EPCs. Further studies are needed to compare these cell types side by side.

The BM provides a permissive microenvironment for a variety of stem cells (including, as we envision, VSELs) circulating in PB during embryonic development to promote their homing to this organ. Molecular analysis of gene libraries established from VSELs revealed that, despite a similar small size, primitive morphology, and expression of surface markers that allow for their purification (Sca-1^+ Lin^- $CD45^-$), these cells are, in fact, somewhat

heterogeneous [63]. We found at least three different types of libraries generated from single, sorted VSELs, and some of these libraries exhibited a strong epiblast- or PGC-like gene expression pattern. In support of such a connection, we observed that murine BM-derived VSELs express several genes that are characteristic of epiblast SCs (*Gbx2, Fgf5,* and *Nodal*) and, more importantly, of germline specification and migrating PGCs (*Stella, Prdm14, Fragilis, Blimp1, Nanos3,* and *Dnd1*) [64, 65]. The expression of some of these crucial genes has subsequently been confirmed by demonstrating the presence of transcriptionally active promoters in these genes. Importantly, we recently observed that BM-residing VSELs respond *in vivo* to stimulation by pituitary and gonadal SexHs and begin to accumulate BrdU [21]. Furthermore, gene expression analysis and immunohistochemical staining confirm that these cells express SexH receptors [21].

Although cells morphologically and phenotypically similar to bone marrow VSELs were found in other tissues, adult BM-residing VSELs probably migrate during development, along with HSCs from sites where fetal hematopoiesis is initiated, to fetal liver and subsequently adult BM [66]. Table 1, reports on early-development stem cells isolated from adult BM and skin that express germline markers are listed [67–73], but their relationship to VSELs requires further study. Nevertheless, these observations support the concept that developmentally early stem cells from embryogenesis could be deposited in adult tissues and that there exists in the stem cell compartment a stem cell continuum beginning with embryonic development and extending into adulthood [24].

The role of parentally imprinted genes in maintaining the quiescence of developmentally early adult stem cells.

As discussed above, evidence has accumulated that adult tissues contain certain early-development stem cells that are endowed with broad trans-germ layer differentiation and multi/pluripotent—for example, VSELs. Nevertheless, to call a given stem cell “pluripotent” requires satisfying both *in vitro* and *in vivo* criteria. For *in vitro* criteria, a pluripotent stem cell candidate has to show undifferentiated morphology, euchromatin, and a high nuclear/cytoplasmic ratio. Such cells should also express markers of pluripotency, such as Oct-4, Nanog, and SSEA, and exhibit bivalent domains in the promoters of genes encoding important developmental, homeobox-containing transcription factors, and female pluripotent stem cells should reactivate the X chromosome. Moreover, such cells should differentiate in appropriate culture conditions into cells from all three germ layers (meso-, ecto-, and endoderm). On the other hand, in vivo criteria for pluripotent stem cells include the ability to complement blastocyst development and grow teratomas in an *in vivo* assay after injection of these cells into immunodeficient mice.

VSELs fulfill the above-listed *in vitro* criteria, despite the fact that they are highly quiescent in culture, and special conditions are needed to differentiate them into various lineages [61, 62, 74–80]. However, VSELs do not fulfill the *in vivo* criteria, as they do not complete blastocyst development and do not grow teratomas [25, 81]. The reason for quiescence of these cells is the modification of expression of certain parentally imprinted genes. Overall, there are ~50–100 paternally imprinted genes in the mammalian genome - expressed from

the maternal or paternal chromosome only, that play an important role in embryonal development. Some of them, for example the tandem gene insulin-like growth factor 2 (*Igf2*)–*H19*, are of particular importance for the totipotential state of the zygote, embryogenesis, fetal growth, and pluripotency of developmentally early stem cells [26, 82–84].

To explain the developmental role of parentally imprinted genes, mammalian development requires proper gene dosage of these genes, which is enabled by their imprinting, so that a single parental allele (maternal or paternal) is expressed in the cell. Therefore, genomic imprinting is an epigenetic program that ensures the parent-of-origin-specific monoallelic transcription of imprinted genes and results in intracellular expression of imprinted genes from only one of the two paternal chromosomes—derived either from the mother or the father [85]. The epigenetics behind expression of imprinted genes is based on the imposition of epigenetic marks by DNA methylation within differentially methylated regions (DMRs), which are CpG-rich *cis*-elements within their loci [26, 82–84]. These epigenetic marks imposed on DMRs in the female germline act on the promoters of imprinted genes, which results in the heritable repression of the maternal chromosomes. By contrast, the imposition of epigenetic marks by methylation of the chromosomes in the male germline does not occur at the promoters, but rather within the intergenic regions (e.g., between the tandem genes at the *Igf2–H19* locus, as shown by black lollypops in Figure 3).

Figure 3A shows that expression of *Igf2* and *H19* genes is regulated by a distal enhancer. Since maternal imprinting at the DMR for this tandem gene is erased (open lollypops) at the maternal (M) chromosome, this site binds CTCF protein (insulator), which forms a physical barrier between *Igf2* and *H19* and thereby prevents the distal enhancer from activating transcription of *Igf2* from the maternal allele. By contrast, the DMR region at the paternal chromosome (P) is methylated (black lollypops), and CTCF cannot bind to the DNA. Thus, the distal enhancer activates transcription of *Igf2* from the paternal allele.

While *Igf2* promotes proliferation, *H19* gives rise to non-coding mRNA that is spliced into several miRNAs that negatively affect cell proliferation. As the result of normal, balanced paternal imprinting in cell nuclei, there is balanced expression of Igf2 mRNA from paternal and H19 mRNA from the maternal chromosome [86].

As mentioned above, erasure of genomic imprints is one of the crucial mechanisms that prevents PGCs and VSELs from proliferation, blastocyst complementation, and teratoma formation [25, 81]. As a result of erasure of the DMR at the *Igf2–H19* locus (Figure 3B), both maternal and paternal DMRs bind insulator protein, and the distal enhancer activates transcription of *H19* from both parental alleles. Cells affected by this epigenetic mechanism do not express insulin-like growth factor 2 (IGF-2), which promotes cell proliferation, and overexpress noncoding H19 mRNA, thereby negatively affecting cell proliferation. This epigenetic change in expression at the *Igf2–H19* locus explains why PGCs and VSELs remain quiescent [25, 87].

To get a full picture of these epigenetic changes, in addition to erasure of imprinting at the *Igf2–H19* locus, murine BM-residing VSELs also erase the paternally methylated imprints

within the DMRs for *RasGrf1*. In parallel, they hypermethylate the maternally methylated DMR for the insulin-like growth factor 2 receptor gene (*IGF2R*). As a result of these changes, VSELs, like PGCs, are resistant to insulin/insulin-like growth factor signaling. Specifically, the changes in expression of imprinted genes lead to a perturbation of insulin/insulin-like growth factor signaling by downregulation of i) IGF-2, which is an autocrine factor involved in proliferation of VSELs, and ii) RasGRF1, which is a GTP-exchange factor (GEF) crucial for signaling from the activated insulin-like growth factor 1 receptor (IGF-1R) and the insulin receptor (InsR). In addition, since the IGF2R serves as a decoy receptor that prevents IGF-2 from binding to IGF-1R, hypermethylation of the DMRs on the maternal chromosome encoding IGF-2R, which leads to overexpression of this gene, has an additional negative effect on IGF-2 signaling in VSELs [88]. Our recent data suggests that a very similar mechanism is also most likely responsible for the quiescent state of human VSELs not only in bone marrow but also in adult tissues. This mechanism, characteristic of PGCs and VSELs [25, 87], keeps them in a quiescent state. As we have shown, the finding that the most primitive stem cells in adult bone marrow are endowed with long-term reconstituting potential [25] has recently been confirmed by another group [89].

In summary, these epigenetic modifications of imprinted loci (including *Igf2–H19, RasGRF1, and IGF2R*) hampers efficient expansion of these cells in *ex vivo* cultures but, on the other hand, prevents them from undergoing uncontrolled proliferation and teratoma formation *in vivo*. Curiously, we noticed that a proper somatic imprinting at this locus could be re-established after exposure of VSELs to nicotinamide.

Conclusions

Evidence has accumulated for the existence of a developmental link between PGCs, VSELs and HSCs, shedding new light on the developmental hierarchy of the stem cell compartment in adult BM. Our group has identified VSELs in adult tissues and demonstrated that epigenetic modification of certain imprinted genes in these cells plays a crucial role in controlling their proliferation. On the other hand, reversal of this imprinting mechanism is crucial to employing these cells in regenerative medicine. Currently, we are testing whether modulation of parental imprinting to activate genes involved in insulin/somatotropins signaling will promote VSEL expansion, as has recently been demonstrated for PSCs derived by parthenogenesis [90]. Our encouraging data indicate that we are able to expand and specify VSELs ~ 3×10^6 in serum free medium in the presence of nicotinamide and cocktail of FSH, LH, BMP-4 and KL [91]. Most important in this chemically defined medium VSELs undergo asymmetric divisions what is an important hallmark of primitive stem cells [92]. Moreover, another team was successful in *ex vivo* expression of VSELs in the presence of the small pyrimido-indole derivative molecule UM177 [93]. In sum, to date a presence of these cells in adult postnatal tissues have been demonstrated by at least 25 independent laboratories [94–97]. There are also reported new strategies to enrich for VSELs from hematopoietic tissues [98, 99].

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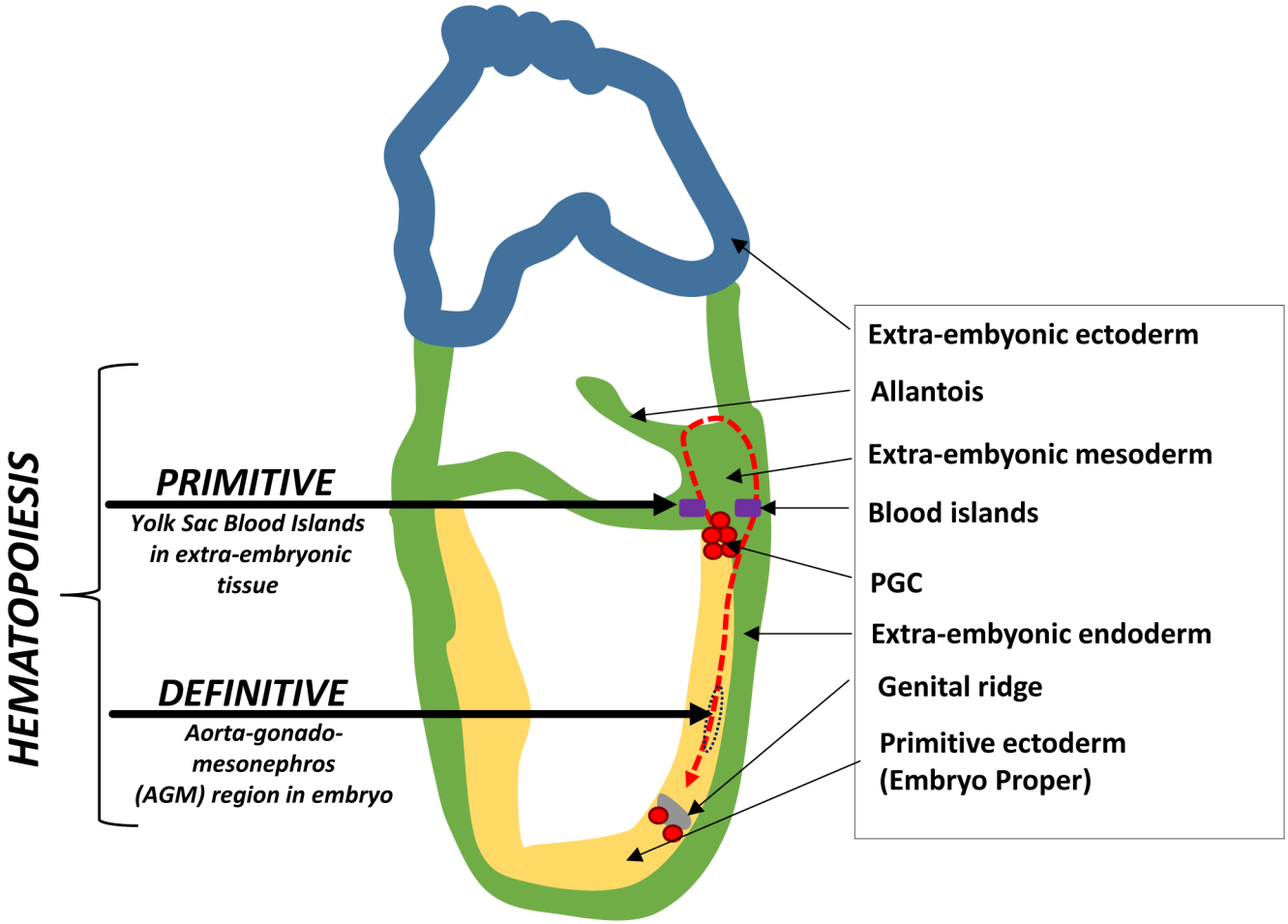

Figure 1. Migration of PGCs and the developmental origin of primitive and definitive hematopoiesis.

The specification of the first primitive HSCs in the yolk sac blood islands as well as the origin of definitive HSCs in the aorta–gonado–mesonephros (AGM) region are chronologically and anatomically correlated with the developmental migration of primordial germ cells in extra- and intra-embryonic tissues. For reasons of simplicity, the developmental difference between the times when primitive and definitive hematopoiesis are initiated is not reflected on this figure by changes in embryo maturation.

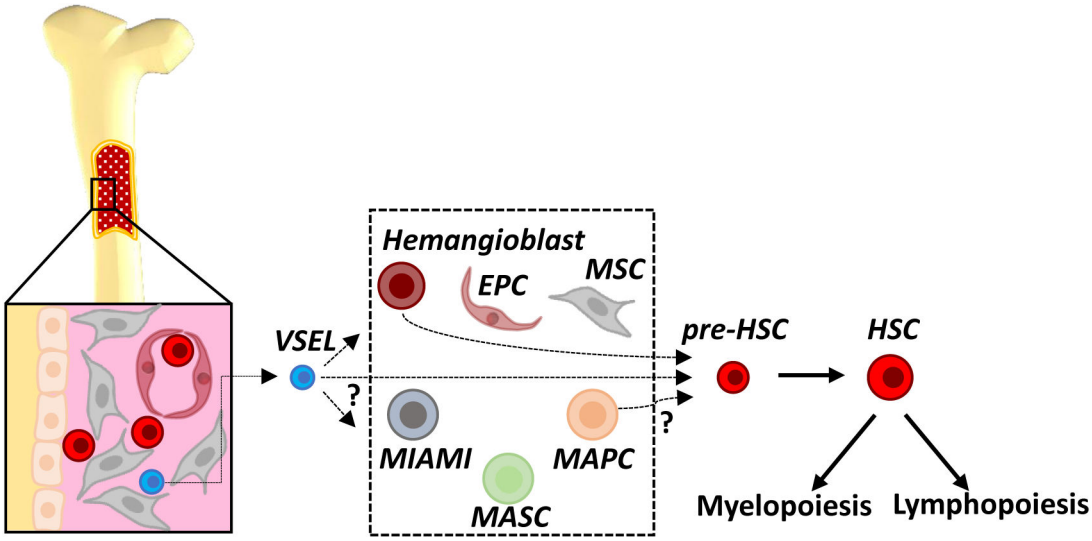

Figure 2. Adult bone marrow as a home for various stem cells.

We propose that VSELs are primitive, small, dormant, stem cells that, upon proper activation, give rise to other, larger multi/pluripotent stem cells identified by other investigators in hematopoietic tissues and may also give rise to hematopoietic/stem progenitor cells, mesenchymal stem cells, and endothelial progenitor cells. Abbreviations: VSEL, very small embryonic-like stem cell, HSC, hematopoietic stem cell, EPC, endothelial progenitor cell, MSC, mesenchymal stroma cells, MASC, multipotent adult stem cell, MIAMI, marrow-isolated adult multilineage-inducible cell, MAPC, multipotent adult progenitor cell.

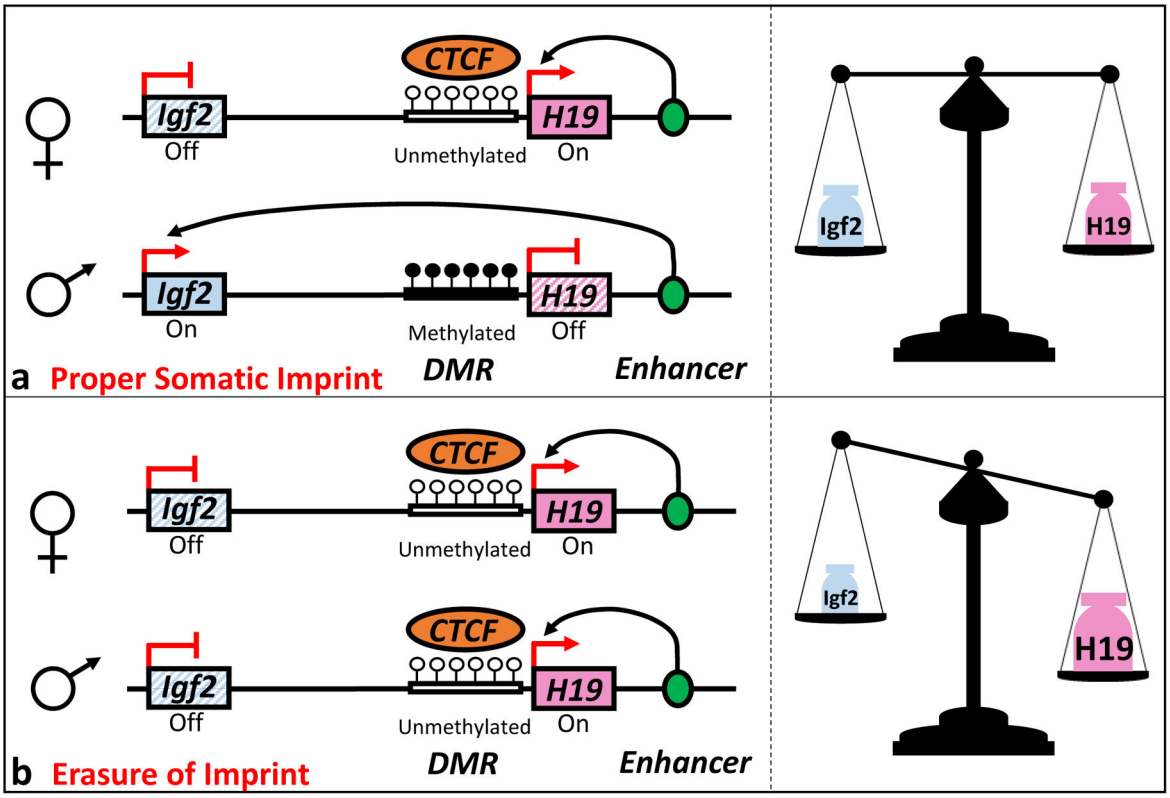

Figure 3. Regulation of expression of the *Igf2–H19* tandem gene.

Panel A. The *Igf2* and *H19* coding regions are separated by a differentially methylated region (DMR) that is unmethylated (open lollypops) on the maternal chromosome (M) and methylated (filled lollypops) on the paternal chromosome (P). Expression of both genes is regulated by a 3' distal enhancer depicted in green. Since the DMR is unmethylated on the maternal chromosome, it binds CTCF, and this prevents activation of the *Igf2* promoter by the distal enhancer. As a result, only H19 mRNA is transcribed from the maternal chromosome (red arrow). By contrast, methylation of the DMR on the paternal chromosome prevents binding of the CTCF insulator protein and allows activation of the *Igf2* promoter by the distal enhancer and transcription of Igf2 mRNA from the paternal chromosome (red arrow). Normal somatic imprinting observed in all somatic cells results in properly balanced expression of Igf2 from the paternal chromosome and H19 from the maternal chromosome.

Panel B. Erasure of imprinting at the *Igf2–H19* locus, as seen in PGCs and VSELs, leads to a situation in which DMRs on the maternal and parental chromosomes both bind CTCF, and the 3' distal enhancer activates transcription of only *H19* from both chromosomes. Therefore, erasure of imprinting at the *Igf2–H19* locus leads to overexpression of proliferation-inhibiting H19 mRNA. We noticed that a proper somatic imprinting at this locus is re-established after exposure of VSELs to nicotinamide.

Table 1.

Selected publications from other authors indicating that stem cells endowed with germline potential reside in postnatal non-gonadal tissues. [100]

Cells endowed with germline markers residing in BM and skin	Reference
Stem cells with germline potential isolated from newborn mouse skin – Oct-4$^+$ cells isolated by FACS from Oct-4–GFP mice, which are able to give rise in vitro and in vivo to early oocytes. Similar cells were also identified in newborn porcine skin.	[67]
Multipotent stem/stromal cells isolated from porcine skin – Oct-3/4$^+$, Nanog$^+$ Sox-2$^+$ cells isolated from porcine skin and adipose tissue and able to differentiate into oocyte-like cells.	[68]
SSEA-1$^+$ murine BM cells – Isolated from murine BM by anti-SSEA-1 immunomagnetic beads. In the presence of bone morphogenetic protein 4 (BMP4), these cells differentiate into Oct-4$^+$Stella$^+$Mvh$^+$ gamete precursors.	[69]
BM-derived germ cell candidates – Oct-4$^+$ Mvh$^+$ Dazl$^+$ Stella$^+$ cells present in BM that may affect the recurrence of oogenesis in mice sterilized by chemotherapy.	[70, 71]
BM-derived male germ cells – Oct-4$^+$ Mvh$^+$ Stella$^+$ cells isolated as Stra8–GFP cells from bone marrow of Stra8–GFP transgenic mice. These murine bone marrow-derived cells express several molecular markers of spermatogonial stem cells and spermatogonia.	[72]
Chicken BM-derived precursors of male germ cells – GFP$^+$ transgenic chicken Oct-4$^+$SSEA-1/3/4$^+$ cells isolated from bone marrow, which give rise to functional sperm after injection into testes.	[73]