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LEADING ARTICLE

Very small embryonic-like stem cells (VSELs) represent a real challenge in stem cell biology: recent pros and cons in the midst of a lively debate

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The concept that adult tissue, including bone marrow (BM), contains early-development cells with broader differentiation potential has again been recently challenged. In response, we would like to review the accumulated evidence from several independent laboratories that adult tissues, including BM, harbor a population of very rare stem cells that may cross germ layers in their differentiation potential. Thus, the BM stem cell compartment hierarchy needs to be revisited. These dormant, early-development cells that our group described as very small embryonic-like stem cells (VSELs) most likely overlap with similar populations of stem cells that have been identified in adult tissues by other investigators as the result of various experimental strategies and have been given various names. As reported, murine VSELs have some pluripotent stem cell characteristics. Moreover, they display several epiblast/germline markers that suggest their embryonic origin and developmental deposition in adult BM. Moreover, at the molecular level, changes in expression of parentally imprinted genes (for example, Igf2–H19) and resistance to insulin/insulin-like growth factor signaling (IIS) regulates their quiescent state in adult tissues. In several emergency situations related to organ damage, VSELs can be activated and mobilized into peripheral blood, and in appropriate animal models they contribute to tissue organ/regeneration. Interestingly, their number correlates with lifespan in mice, and they may also be involved in some malignancies. VSELs have been successfully isolated in several laboratories; however, some investigators experience problems with their isolation.

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INTRODUCTION

Despite vigorous effort and substantial financial investments, we still do not have in hand a pluripotent stem cell (PSC) that can be safely and efficiently employed to regenerate damaged tissues and organs. Despite a lot of promises, it is difficult even in animal models to demonstrate, in a reproducible way, that any of the proposed murine PSCs can rebuild organs, contributing not only to fragments of parenchyma but also to vessels, nerves and lymphatics. Therefore, taking into consideration the huge interest and expectations of the public, we should pursue all possibilities and alternative approaches to finding such cells, combine our efforts without hostility and unnecessary competition and leave aside financial interests related to patents, company stock and funding by private investors or government agencies. If embryonic stem cells derived by therapeutic cloning in humans work and are safe, why not try them?[1] Similarly, if induced PSC technology turns out to be effective and safe, why not try this technology in the clinic?[2–5] But why in this competition are there so many attempts to condemn stem cells isolated from adult tissues? In fact, because of their safety, stem cells isolated from adult tissues are the only ones that have so far been tested in clinical settings. However, we are aware and we have clearly stated several times that many of the positive effects of adult stem cell therapies are related to their paracrine effects,[6–10] but at the same time, we cannot exclude other more direct mechanisms.[6,11–13]

There has been much debate of late challenging the existence of pluripotent VSELs, these cells which were first isolated in my (MJ Ratajczak's) laboratory 10 years ago,[14,15] and since have been experimentally confirmed by several independent groups.[16–24] Scientific writers fuelled this dispute by mediating their personal views through highly respected journals.[25,26] Very unfortunately we found many errors and inaccuracies among their accusations. In truth these critical comments were recruited from a rather narrow group of investigators.

These investigators, we fear, sadly had possible vested financial interests in induced PSC and embryonic stem cell technology, a very competitive topic. In brief, the small nucleus of attacking scientists declared that VSELs, like other stem cells isolated from adult tissues, have no future in regenerative medicine, and in fact are artifacts. What is absolutely deplorable in our minds is the claim that clinical trials based on VSELs were a fake, or at least were not justified. We cannot help thinking that this was an attack on a grant allotted to investigators working on VSELs through a peer review system by the National Institute of Health and Department of Defense in the United States.

In the wake of these claims and despite the fact that VSELs have been successfully isolated in several laboratories worldwide, three recent papers published in open-access journals, PLoS One,[27,28]

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and the newly created journal Stem Cell Reports,[29] reported unsuccessful attempts to isolate these cells. The unprecedented furor that has been generated around this topic makes one wonder what is really going on? We prefer not to comment on the insinuation that the Vatican is using VSELs to try to control science.[25,26] Some of us are Catholic, like many others, and yes, we have started this research through curiosity. We are firm believers in science and we shall once again try to gather all the facts in our possession to incorporate in this article crucial evidence about the existence of VSELs.

We will review the most important concepts supporting the existence of VSELs in adult tissues and address the evidence against VSELs, and why some have experienced difficulty in purifying these cells.

FROM STEM CELL PLASTICITY TO VSELS

The concept that adult bone marrow (BM), mobilized peripheral blood and umbilical cord blood (UCB) harbor early-development stem cells has been proposed by our team as an alternative to the plasticity or trans-dedifferentiation theory of hematopoietic stem cells (HSCs),[30,31] which claimed that HSCs can 'change their spots' and give rise to other non-HSCs.[32–34] Pursuing an alternative explanation of stem cell plasticity, we proposed that adult hematopoietic tissues contain a population of early-development stem cells,[30,31,35] and we focused on isolation and characterization of these cells at the single-cell level.

In fact, there are numerous reports demonstrating that in adult murine and human tissues there are rare stem cells that express early-development markers and are endowed with broader differentiation potential.[36–44] These stem cells have been given various names, depending on the assays by which they were identified. In most of these reports, the stem cell that differentiated into cells from different germ layers has not been directly identified at the single-cell level. Thus, there was always the unanswered question: what stem cell is ultimately responsible for all these multitissue differentiation phenomena? We believe that many of us studying stem cells residing in adult tissues that display early-development markers and show broader differentiation potential are in fact looking at similar, overlapping populations of stem cells that can be quiescent[44] but, if activated, are able to expand into different lineages. These cells reside at different levels of germ layer/tissue specification and can be detected if appropriate experimental tools are employed.

In our work, we assumed from the beginning that the most primitive stem cells would be small and quiescent and that in BM they would be more primitive than classical hematopoietic stem cells or mesenchymal stem cells, positioned at the top of the developmental hierarchy of these cells. This has, in fact, been confirmed in recent publications.[16,18,45,46] Moreover, in well-controlled experiments, murine VSELs were demonstrated to give rise to lung alveolar epithelial cells,[17] whereas rat VSELs differentiated into cardiomyocytes and endothelial cells.[47] Finally, a corresponding population of small embryonic-like stem cells identified in human and murine ovaries and testes have been shown by two independent groups to have the potential to give rise to gametes,[19,20,24,48] which be discussed later in this article.

To summarize, the whole concept of the origin and existence of VSELs in adult tissues is based on the following theses:

- Adult tissues, including murine BM, harbor a population of very rare stem cells with multiple tissue differentiation potential, indicating that the hierarchy of the stem cell compartment in adult tissues and BM needs to be revisited.
- These dormant, early-development cells show several epiblast/germline markers that suggest their embryonic origin/deposition and relationship to early epiblast-derived migrating primordial germ cells (PGCs, Figure 1a). Epigenetic changes in imprinted genes, such as erasure of the maternal type of imprinting at differentially methylated regions, which are specific marks in PGCs to prevent them from undergoing teratoma formation,[49–53] are also responsible for the quiescence of VSELs in adult tissues. As imprinted genes (for example, those encoded at the Igf2–H19 locus) are involved in insulin/insulin-like growth factor signaling (IIS), epigenetically regulated resistance of VSELs to IIS regulates their quiescent state.[54]
- However, although these cells can be activated and mobilized into peripheral blood during several known physiological emergencies,[55–60] they may also be involved in maintaining the pool of tissue-committed stem cells. The number of VSELs correlates with lifespan,[61–63] and their potential role in tumorigenesis requires further attention.[64,65]

Murine BM-purified VSELs possess several *in vitro* features expected from PSCs, such as a characteristic morphology in transmission electron microscopy (a high nuclear/cytoplasmic ratio with a thin rim of cytoplasm, the presence of euchromatin and few mitochondria), and express Oct-4 and Nanog at the mRNA and protein levels,[14] which has received further confirmation by promoter methylation studies showing their association with histone codes that promote transcription.[49] Furthermore, VSELs express bivalent domains at promoters of developmentally important transcription factors,[66] and female VSELs reactivate the X chromosome.[67] In appropriate culture systems, these cells can also differentiate into cells from different lineages. Murine VSELs, however, do not form teratomas and do not complete blastocyst development, which is a key feature of classical PSCs, such as embryonic stem cells or induced PSCs. However, this lack of pluripotentiality of murine VSELs should not be surprising, because early-development stem cells present in the adult body should be well protected from the risk of teratoma formation.

THE DEVELOPMENTAL ORIGIN OF VSELS EXPLAINS THE EPIGENETIC CHANGES REGULATING THE EXPRESSION OF PATERNALLY IMPRINTED GENES THAT GOVERN THEIR QUIESCENCE IN ADULT TISSUES

Significant effort has been devoted to characterizing VSELs at the molecular level in order to determine their developmental origin. In studies performed on highly purified double-sorted VSELs isolated under steady-state conditions from murine BM, we observed that these cells highly express, at the mRNA and/or protein levels, genes involved in both specification of the epiblast (for example, *Stella*, *Prdm14*, *Fragilis*, *Blimp1*, *Nanos3* and *Dnd1*) and PGCs.[68,69] The PGC-specific genes, such as *Dppa2*, *Dppa4* and *Mvh*, are characteristic of late-migratory PGCs. The expression of some of these crucial genes has been confirmed subsequently by demonstrating the presence of transcriptionally active promoters.[68] Based on the molecular similarity between VSELs and PGCs, we presented the hypothesis that one of the mechanisms that keep VSELs quiescent in adult tissues is similar to the one that governs quiescence of PGCs and is based on epigenetic modification of paternally imprinted genes (Figure 1b).

Pursuing this hypothesis, we observed that VSELs, like PGCs migrating during embryonic development, modify the imprinting of some early-development parentally imprinted gene loci, including Igf2–H19, which results in their resistance to IIS, and by modifying imprinting and KCNQ1/$p57^{Kip2}$ upregulate the cyclin-dependent kinase inhibitor $p57^{KIP2}$.[49] We also observed that, in addition to changes in expression of imprinted genes, VSELs express several miRNAs that attenuate IIS signaling in these cells (for example, mir681, mir470 and mir669b) as well as upregulate the expression of $p57^{KIP2}$ (for example, mir25.1, mir19b and mir92; M Maj, manuscript in preparation).

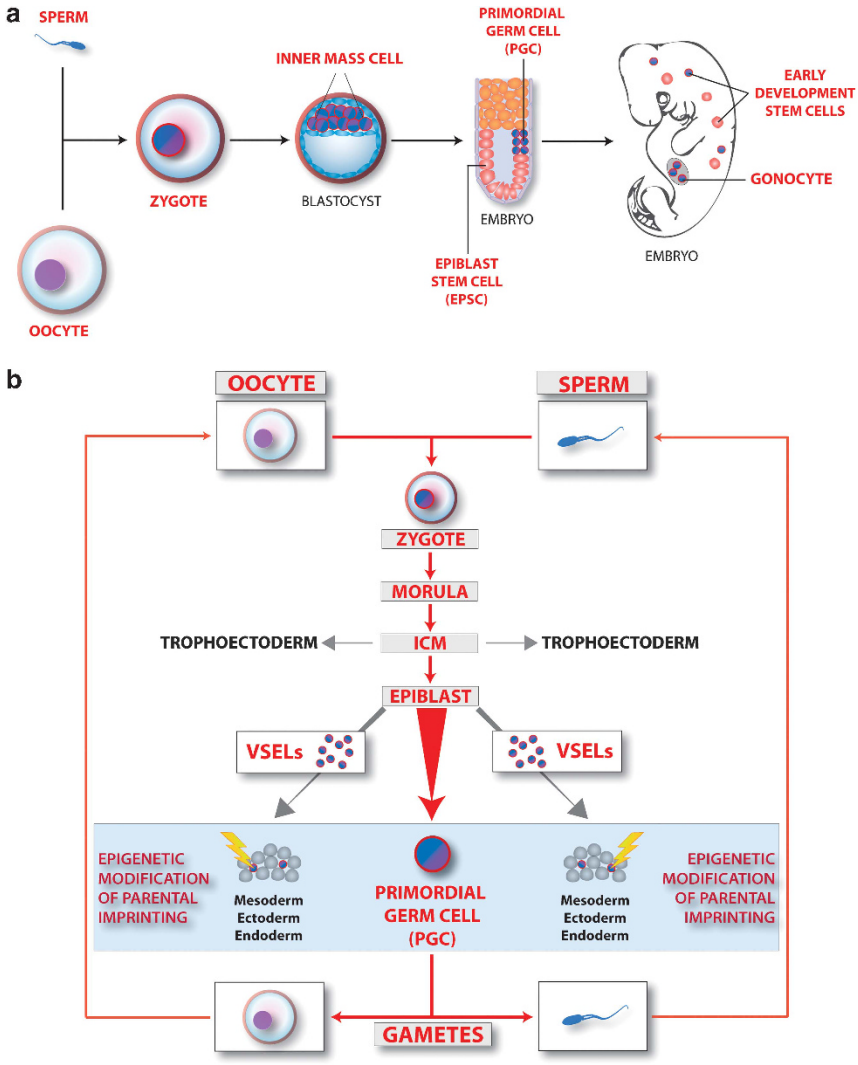


Figure 1. The overall concept of a presence of developmental early epiblast/germline-derived stem cells in adult tissues. (**a**) Retention of germline potential during ontogenesis. Cells with germline potential are shown in blue. The earliest and the most primitive cell in the germline is the totipotent zygote. The germline potential is subsequently retained during development in Oct-4$^+$ cells located in the inner cell mass cells (ICMs) of the developing blastocyst, epiblast stem cells (EPSCs), primordial germ cells (PGCs) and gonocytes in gonads. Epiblast/germline potential could also be retained in rare early-development small VSELs deposited during development in peripheral tissues as founders for more differentiated monopotent tissue-committed stem cells. Some of these small cells express Oct-4 (shown in blue). (**b**) The cycle of life—from zygote to germ cells. From a developmental and evolutionary point of view, the germline (shown by red arrows) carries the genome (nuclear and mitochondrial DNA) from one generation to the next, and all somatic cell lines bud out during ontogenesis from the germline to help germline cells accomplish this mission effectively. The germline potential is established in the fertilized oocyte (zygote) and subsequently retained in the morula, inner cell mass of the blastocyst (ICM), EPSC, PGCs and mature germline cells (oocytes and sperm). The first cells that bud out from the germ lineage are trophoectodermal cells, which give rise to the placenta. Subsequently, during gastrulation, EPSCs are a source of PSCs for all three germ layers (meso-, ecto- and endo-derm) and PGCs. We hypothesize that at this stage some EPSCs and PGCs are deposited as Oct-4$^+$ VSELs in tissues developing from meso-, ecto- and endo-derm (blue circles). Blue box and yellow arrows pointing at VSELs indicate mechanism based on epigenetic modification of parentally imprinted genes (for example, at Igf2–H19 and KCNQ1p57^{Kip2} loci) that keeps these early-development cells quiescent in adult tissues. A similar mechanism based on erasure of imprinting also regulates the quiescent state of PGCs.

More importantly, to support a link between PGCs and VSELs as precursors of long-term repopulating HSCs (LT-HSCs),[45,70,71] we observed that VSELs and HSCs express mRNA for several pituitary and gonadal hormone receptors as well as highly express Sall4, an early-development marker shared by germ and hematopoietic cells.[72,73] Finally, in direct *in vivo* and *in vitro* experiments, we confirmed that the quiescent population of BM-residing VSELs, like HSCs, expands in response to stimulation by androgens (danazol) and pituitary gonadotropins such as pregnant mare serum gonadotropin (PMSG), luteinizing hormone (LH), and follicle-stimulating hormone (FSH). In support of this concept, we observed that a 10-day administration of all these sex hormones directly stimulated expansion of VSELs and HSCs in BM, as measured by an increase in the total number of these cells in BM (~2–3x) and enhanced 5-bromodeoxyuridine (BrdU) incorporation (the percentage of quiescent BrdU$^+$Sca-1$^+$Lin$^-$CD45$^-$ VSELs increased from ~2% to ~15–35%, and the percentage of BrdU$^+$Sca-1$^+$Lin$^-$CD45$^+$ HSCs increased from 24% to 43–58%) (K Mierzejewska, manuscript in preparation).

Overall, our 2008 paper demonstrated for the first time that the quiescent state of the most primitive stem cells in murine BM can be regulated by epigenetic changes of imprinted genes,[49] as seen

in the case of PGCs (Figure 1b). This has been somehow very recently confirmed in a paper by Venkatraman *et al.*,[74] who found that most primitive LT-HSCs in murine BM are kept quiescent by the maternal type of imprinting at the Igf2–H19 locus, which confers resistance to IIS. As mentioned above, we provided evidence that VSELs may become specified into LT-HSCs.[45,46]

RECENT DATA FROM INDEPENDENT LABORATORIES THAT SUPPORT THE EXISTENCE OF VSELS

As mentioned above, several primitive cells with the characteristics of pluripotency or multipotency were identified in adult murine and human BM, UCB and adult solid organs that were enzymatically processed to get cell suspensions for analysis. Such cells, more or less depleted of lineage-positive cells,[36–39,75–78] were cultured and expanded *in vitro*, usually as adherent cell populations, and if they expanded into cells from different tissues, they were assigned various operational names such as multipotent adult stem cells,[36] multipotent adult progenitor cells,[39] MIAMI cells,[37] multilineage-differentiating stress-enduring (Muse) cells[76,79] or unrestricted somatic stem cells.[38] Unfortunately, the phenotype of the most primitive stem cell that initiated these cultures has never been clearly described. In addition to assays based on *in vitro* cultures, other isolation strategies have also been employed, for example, an interesting population of small cells (ELH stem cells) isolated from murine BM by elutriation (E), lineage depletion (L) and the ability to home (H) to BM has been described, which is able to differentiate into epithelial cells and HSCs.[80–82] Another group reported the presence of small cells (known as 'spore-like stem cells') in adult mammalian tissues that are able to differentiate into cells from all germ layers and have been isolated from adult mammalian tissues.[83]

Moreover, several recent reports based on fluorescence-activated cell sorting multiparameter sorting strategies were published that supported the existence of small, primitive VSELs and VSEL-like cells in adult tissues (the most important are listed in Table 1). For example, murine BM-sorted Sca-1$^+$Lin$^-$CD45$^-$ VSELs have been shown to give rise to type 2 pneumocytes, which produce lung surfactant protein after transplantation into surfactant-deficient mice.[17] Furthermore, small SSEA1$^+$Lin$^-$CD45$^-$ cells that express Oct4$^+$ sorted from rat BM gave rise *in vivo* to cardiomyocytes and endothelial cells in an experimental model of rodent acute myocardial infarction.[47] Furthermore, Sca-1$^+$Lin$^-$CD45$^-$ VSELs cells from murine BM[16] or human mobilized peripheral blood[18] cells expressing the SSEA4$^+$CD133$^+$CXCR4$^+$Lin$^-$ and CD45$^-$ phenotype and isolated by fluorescence-activated cell sorting formed murine and human bones, respectively, when embedded in gelatin sponges and implanted into immunodeficient mice. This bone-forming activity exceeded the activity of the other populations of BM-purified cells that were evaluated using the same assay. Based on this finding, it has been proposed that these BM- and mobilized peripheral blood-purified VSELs are at the top of the hierarchy for the mesenchymal and endothelial lineages.[16,18] Cells similar to BM-derived VSELs have been reported to reside in the ovarian surface epithelium in postmenopausal ovaries[19,48] and normal testes[20] and were able to differentiate into gamete-like cells. As reported, these gonadal VSELs express nuclear OCT-4A, whereas the immediate descendants of these cells express cytoplasmic OCT-4B isoform.[19,20]

Furthermore, human mobilized peripheral blood VSELs have been successfully purified by other researchers,[18,21] and very small Oct4$^+$Sox2$^+$ cells corresponding to UCB-derived VSELs described by us[15,46] were purified from UCB by other investigators, who described them as a population of UCB-derived VSELs[23,84] or UCB-derived embryonic-like stem cells.[85,86] Interestingly, these cells were able to differentiate *in vitro* into neural progenitor cells.[85] Finally, a corresponding population of primitive Oct4$^+$ stem cells that resembles VSELs and was named omnicytes was also described as circulating in UCB and was capable of migrating into the maternal circulatory system.[87]

In addition to the cells listed in Table 1, in a recent report, small cells with some VSEL markers were also identified in murine neonatal retina.[88] In another study, small non-hematopoietic lineage-negative (Lin$^-$) cells isolated from adult BM by elutriation (Fraction 25 or Fr25) were involved in retinal regeneration following the induction of anterior ischemic optic neuropathy and optic nerve crush injury in a rodent model.[89] In addition, a similar population of small non-hematopoietic CD45$^-$ stem cells harvested from BM via elutriation was recently shown to differentiate *in vivo* into functional insulin-producing cells in chemically induced diabetic mice.[90] Similar small cells isolated by elutriation were reported in the past to give rise to LT-HSCs.[80]

Thus, VSELs and VSEL-resembling cells have recently been purified in several laboratories worldwide, and we can expect that the coming years will bring more new information regarding their biology and *in vitro* and *in vivo* differentiation potential.

THE 'DENIERS', OR WHY SOME INVESTIGATORS WERE UNSUCCESSFUL IN PURIFYING VSELS

Although VSELs have been successfully isolated in several independent laboratories, three groups have recently reported that they were not successful in the isolation of VSELs.[27–29] These events sparked an excessive reaction from some scientific writers, who published their personal views on the topic using highly regarded journals as a forum.[25,26] We will discuss here from the scientific point of view the problems that we see with the sorting strategies employed by the three groups. In our opinion, most of the difficulties that they encountered[27–29] could have been entirely eliminated or greatly minimized if they had first contacted us for help and discussion, which is always a good practice in science.

In the first paper, although Miyanishi *et al.*[29] tried to follow our isolation protocols for VSEL sorting that were established for mice BM, they were eventually not successful in VSEL isolation, and these small cells were somehow lost, either during BM preparation or in the sorting procedure. This occasionally happens even in our experienced hands and in the hands of other groups that sort these very rare cells. Thus, based on our broad experience in VSEL isolation from several animal species[14,61,91–96] and human specimens,[15,46,55,97] we will discuss few main steps in the gating strategy described by Miyanishi *et al.*[29] that may explain the VSEL loss and subsequent negative results that this group reported.

First, in our experience, in order to isolate VSELs effectively, a gate including Lin$^-$/Sca-1$^+$ cells should be set up strictly for Lin-negative objects. Although we understand that the authors' intention was to enlarge the gate into Lin^{dim} objects to increase VSEL yield, we have found that such a step may in fact greatly enrich the sorted population in erythroblasts and thus significantly dilute the VSELs.[29] This concern should be taken under consideration, especially as the authors do not provide detailed information about their red cell lysis conditions, buffers, cell washing and the centrifugation of BM cells before VSEL separation.[29] These steps are crucial for successful VSEL isolation and greatly affect the potential presence of cell debris and artifacts in BM samples used subsequently for VSEL sorting.

This potential dilution effect could have critically affected further analytical tests performed on sorted samples,[29] such as: (1) gene expression analysis including Oct-4 (if the VSEL-derived transcripts are limited, the detected expression could be low or none) and (2) VSEL functionality in culture (where the initial VSELs aggregation step is crucial for expansion on both C2C12 and OP-9 cells and the number of VSELs in the seeded material is critical). Similarly, including CD45^{int}/Lin$^-$/Sca-1$^+$ cells in the sorted

Table 1. Selected reports from other groups on stem cells that are attributable to VSELs

Cells as originally named in the paper	*Isolation procedure and properties as described in the original paper*	*References*
ELH cells	Small cells, ~5 μm in diameter, isolated by elutriation and FACS sorting or by elutration (E), lineage depletion (L), and recovered after homing (H) to BM. Give rise to long-term reconstituting hematopoietic stem cells (LT-HSCs) and epithelial cells.	80–82
Spore-like stem cells	Small cells, ~5 μm in diameter, isolated from various murine tissues, resistant to freeze/thawing, expressing Oct-4, and showing broad differentiation. Isolation procedure not revealed.	83
Small non-hematopoietic Sca-1$^+$ Lin$^-$ CD45$^-$ cells	Isolated by FACS from murine BM give rise to type II pneumocytes, producing surfactant in lung alveolar epithelium. Recently, these cells have been confirmed to be VSELs.	17,106
Rat VSELs	Isolated by FACS from rat bone marrow as SSEA$^+$ Lin$^-$ CD45$^-$ cells that express Oct-4 and are endowed with cardiomyogenic and endothelial potential.	47
Human PB-derived VSELs	Isolated by FACS as Sca1$^+$Lin$^-$CD45$^-$ from murine BM or as SSEA-4$^+$ CD133$^+$ CXCR4$^+$ Lin$^-$ and CD45$^-$ from human PB—described as being at the top of the hierarchy for the mesenchymal lineage. Formed bone fragments in immunodeficient mice.	18,21
Ovarian VSELs	Small Oct-4$^+$ SSEA$^+$ cells isolated by FACS from ovarian surface epithelium (OSE) from mice and humans—precursors of female gametes. Human OSE-derived VSELs were characterized extensively by gene array for mRNA expression.	19,24,48,107
Testicular VSELs	Small Oct-4$^+$ SSEA$^+$ cells identified in murine and human testes—precursors of male gametes	20
Embryonic-like stem cells from UCB	Small CD45$^-$, CD33$^-$, CD7$^-$, CD235a$^-$ pluripotent stem cells (2–3 μm in diameter) coexpressing embryonic stem cell markers, including Oct4 and Sox2, and able to differentiate into neuronal cells.	85,86
UCB VSELs	Small Oct-4$^+$, SSEA-4$^+$, Nanog$^+$, Sox-2$^+$, Rex-1$^+$, and Tert$^+$ cells isolated from UCB.	15,23,46,84
Omnicytes	Small Oct-4$^+$ stem-cells identified in UCB, able to establish fetal–maternal chimerism.	87

Abbreviations: BM, bone marrow; FACS, fluorescence-activated cell sorting; PB, peripheral blood; UCB, umbilical cord blood; VSEL, very small embryonic-like stem cell.

population of CD45$^-$/Lin$^-$/Sca-1$^+$ cells (containing, in fact, VSELs) could result in VSEL dilution and eventually negative experimental outcomes in any type of functional experiment.[29] This might have been avoided if the authors had discussed the sorting and experimental pitfalls with our groups before performing their experiments.

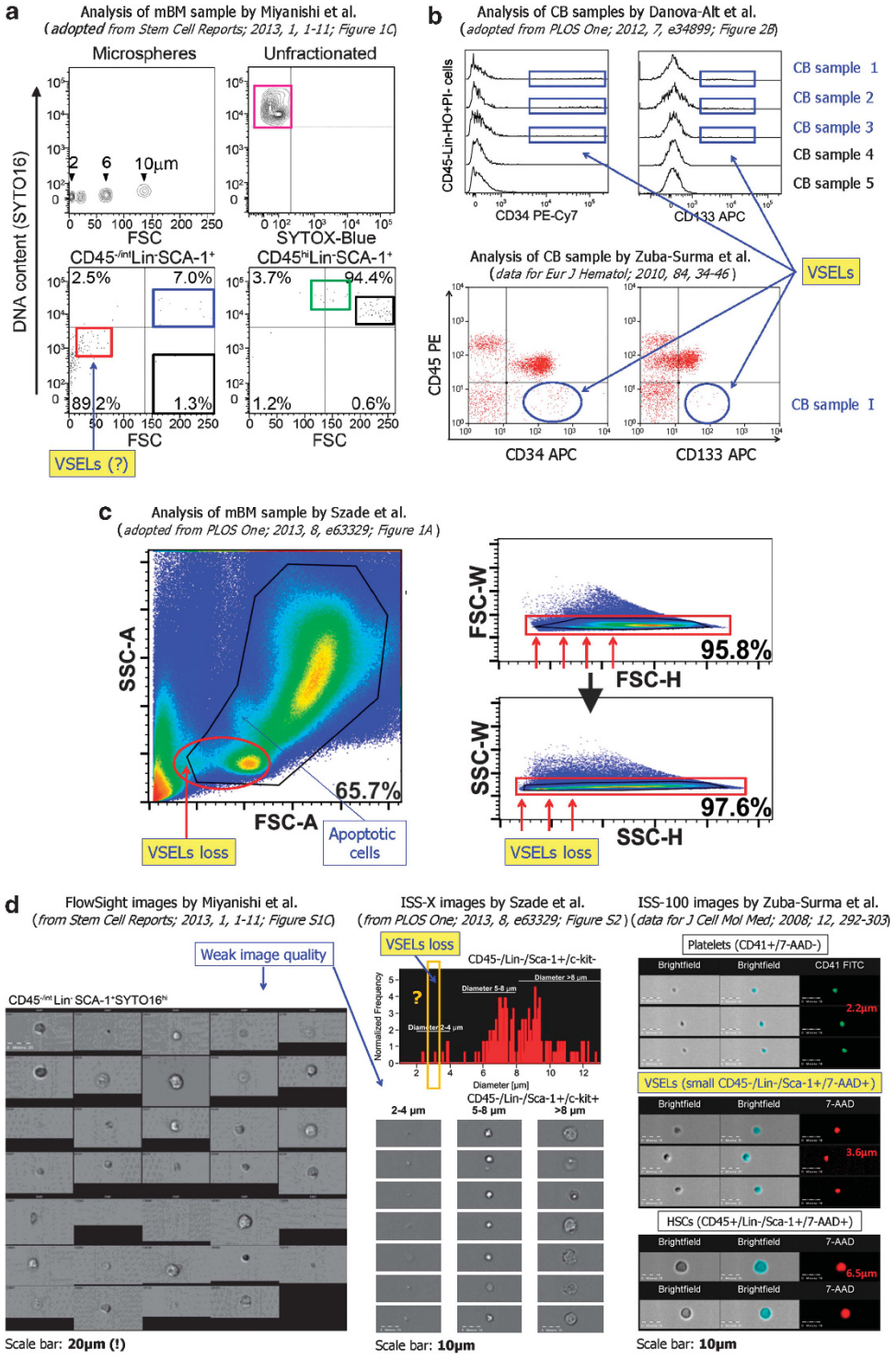
Other important steps in sorting and analysis that we would recommend doing differently than the authors are BM cell staining with Syto16 and its interpretation.[29] The authors use Syto16 as a dye indicating diploid cells, when in fact it may also give a signal related to cytoplasmic contents and therefore cannot be equated with DNA-selective dyes such as 4′,6-diamidino-2-phenylindole (DAPI) or Hoechst 33342, as indicated by the manufacturer.[98] The manufacturer specifically states: 'The SYTO dyes do not act exclusively as nuclear stains in live cells and should not be equated with DNA-selective compounds such as DAPI or Hoechst 33 342 which stain nuclei in live animal cells. Eukaryotic cells incubated with SYTO dyes generally show cytoplasmic or mitochondrial staining, as well as nuclear staining'. This was confirmed in recent publication.[99] As cell ploidy is defined by DNA content, clearly SYTO dyes cannot be used as ploidy markers, and the accuracy of the authors' ploidy estimation can be called into question. Despite this obvious mistake, based on the cell distribution shown in Figure 2C in the original paper by Miyanishi *et al.*,[29] the authors conclude that 'VSEL candidates' consist of the large cells (>10 μm) with high Syto16-related signals (interpreted by the authors as normal, 2n cells, original Figure 1C[29] and shown here as Figure 2a, blue box). On the contrary, we consider the fraction of smaller cells (FCS^{low}) appearing as Sytox16^{dim} cells (originally Figure 1C[29] and shown as Figure 2a in this article, red box) to represent potential VSELs because of their well-known low cytoplasmic content and greater resistance to DNA staining, which has been characteristic of these cells in our experience as well as that of other groups.[17,19,96,100] Unfortunately, as Miyanishi *et al.*[29] decided to sort the Syto16^{bri} cells (which most likely belong to the CD45^{int} subpopulation of progenitors or erythroblasts in the analyzed material) as 'VSEL candidates', we are not surprised that they were unsuccessful in isolating VSELs in subsequent steps. Because of this crucial mistake and their resultant focus on the wrong population, the authors' statement '*This indicated that our VSEL candidates were the relatively larger cells in the population analyzed, unlike the VSELs described in previous reports* (Kucia *et al.*, 2006; Ratajczak *et al.*[45]; Wojakowski *et al.*[55]; Zuba-Surma *et al.*[29,94–96]) is not supported by any evidence.

The Sytox16-related signal may vary between cell populations, raising questions about its discriminatory role for diploid cell identification, and this problem is also clearly visible in the example of the CD45^{hi}/Lin$^-$/Sca-1$^+$ cell fraction (located on the border of the ±10-μm bead size, originally Figure 1C[29] and shown as Figure 2a in this article, green box), which is close to a half log unit brighter than the majority of FSC^{hi}/CD45^{hi}/Lin$^-$/Sca-1$^+$ cells.[29] We would like to ask: what does this mean? Does Syto16 indicate that these cells are proliferating, 3n, or are they in fact 2n and all fractions that the authors interpret as diploid have indeed a lower DNA content? There must be more controls and more cautious interpretation in such analysis.

Importantly, staining with DNA-binding dyes or chromosome-specific probes is not a reliable assay for investigating DNA status, karyotype and DNA stability of adult stem cells, embryonic stem cells and also induced PSCs. In our opinion, global genomic cytogenetic arrays should be performed to confirm normal genetic status and thus the safety of such cells.

In the second of the 'con' papers, Danova-Alt *et al.*,[27] based on their uncertain-quality fluorescent *in situ* hybridization staining with probes directed to only selected chromosomes, concluded that human UCB-derived CD45$^-$/Lin$^-$/CXCR4$^+$ cells (which they considered wrongly to be VSELs) display a high degree of aneuploidy. In contrast, we verified the karyotype of human UCB-derived CD45$^-$/Lin$^-$/CD133$^+$ cells (which we in fact consider to be a VSEL-enriched population) by employing a global genome cytogenetic array (CytoScan 750K, Affymetrix, Santa Clara, CA, USA) and definitively confirmed their normal, diploid status, with neither chromosomal aberrations nor small deletion/insertions in any gene (E Zuba-Surma, manuscript in press). Importantly, as mentioned above, Danova-Alt *et al.*[27] focused on the subset of CD45$^-$/Lin$^-$/CXCR4$^+$ UCB cells that we indicated as being least enriched in VSELs. Based on our data collected within the past few

years, we clearly suggested that primitive Oct-4$^+$, SSEA-4$^+$ cells should be sought within the much rarer fraction of CD45$^-$/Lin$^-$/CD133$^+$ cells.[46,97] Unfortunately, Danova-Alt *et al.*[27] disregarded our recently published results and followed an incorrect population that we initially isolated 5 years ago and then further studied.[15,46,97] Interestingly, although these authors concluded that CD45$^-$/Lin$^-$ cells from UCB essentially lack CD34 and CD133 antigens, these cells are visible even in their histograms (originally Figure 2B[27] and shown as Figure 2b in this article, upper panel), but the authors inexplicably overlooked the rare objects, including the VSEL fraction,[27] which we clearly indicated in our analysis done on dot plots (Figure 2b, lower panel). It is clearly apparent that both CD45$^-$/Lin$^-$/CD34$^+$ and CD45$^-$/Lin$^-$/CD133$^+$ cells are present in at least some UCB samples analyzed by Danova-Alt *et al.*,[27] which are indicated in their original histograms by blue boxes marking rare objects corresponding to both populations (Figure 2b, UCB samples 1, 2 and 3). Another important technical problem is that the authors instead of using for CD133 staining as



we recommended in our original protocols antibodies against CD133/1 epitope[100] employed not optimized for VSEL isolation an anti-CD133/2 eiptope antibody (clone: 293C3).

Similarly, in the third of the 'con' papers, Szade *et al.*[28] went a step further and tried to set up an entirely new protocol for isolation of murine VSELs, discounting our already published strategies, and eventually sorted a fraction distinctly different from very small CD45$^-$/Lin$^-$/Sca-1$^+$ cells, thus presumably losing the VSELs during isolation. Using their *de novo* protocol, these authors[28] made several cardinal mistakes, including: (1) setting up an enlarged input gate on the forward scatter (FSC) vs side scatter (SSC) plot that, on one hand, included granulocytes and apoptotic cells, resulting in enrichment of sorted fractions with artifacts reflected in further analyses (for example, Annexin V binding) and, on the other hand, excluded some of the critical very small objects from further sorting (originally Figure 1A[28] and shown as Figure 2c in this article); (2) losing very small objects further by cutting them out during gating for 'singlets' (originally Figure 1A[28] and shown as Figure 2c in this article); (3) focusing on selection markers that were not proven to be expressed on VSELs (for example, c-kit); and (4) a selective focus on some populations and discarding other fractions (containing potentially VSELs) based on a superficial interpretation of results such as Annexin V binding.[28] For instance, the entire fraction of CD45$^-$/Lin$^-$/Sca-1$^+$/c-kit$^-$/KDR$^-$ was excluded by the authors from further sorting because it was deemed 'apoptotic' (Figure 2[28]), although it most likely contained not only real Annexin V$^+$/FSC^{low}/SSC$^{dim/hi}$ apoptotic objects, but also VSELs. Importantly, we have found that healthy normal cells (including VSELs and HSCs) may bind Annexin V following lysis of red blood cells because of microvesicle/microparticle release and posphatydylserine transfer to the membranes of the normal cells.[101] Thus, not all Annexin V$^+$ objects should be interpreted as 'apoptotic', as these may sometimes represent normal, functional cells.[101]

In this regard, even though Miyanishi *et al.*[29] tried to follow our protocols, we noticed several other flow cytometric concerns when analyzing the identification and sorting approaches used by this group.[29] For instance, (1) how is it possible that, following red blood cell lysis, the unfractionated BM cell population does not contain any Syto16$^-$/Sytox-Blue$^+$ objects, and all events are shown to be uniformly nucleated or 2n (based on the authors' Sytox-16 signal interpretation) healthy cells (originally Figure 1C[29] and shown as Figure 2a in this article, magenta box), whereas Syto16$^-$/Sytox-Blue$^+$ objects are shown in subfractions of BM cells in the same analysis (*how much of this analysis is reliable?*) or (2) why are there, in fact, so few cells indicated in the size region ~4–10 μm in the original Figure 2C (shown by an arrow in Figure 1 in this article), although this population was much more prominent in the analysis shown in the original Figure 1 (shown as an example here in Figure 2b)—*are these vast numbers of cells resembling VSELs lost following Sytox16 staining or software compensation?* Answers to these and several other questions are crucial for the academic community to understand the discrepancies in proper VSEL identification and sorting encountered by Miyanishi *et al.*[29] as well as other groups struggling with VSEL isolation.[27,28] We plan to highlight these methodological aspects in more detail in a separate paper focusing exclusively on flow cytometric pitfalls and techniques in rare stem cell characterization, including for VSELs.

Moreover, Miyanishi *et al.*[29] focused particularly on VSEL cell-size evaluation (originally shown as Figures 1 and 2, and Figure S1 in Miyanishi *et al.*[29]). They, for instance, compared the BM cell distribution with bead standards on different sorting machines (both Aria (BD Biosciences, San Jose, CA, USA) and MoFlo (Beckman Coulter, Inc., Brea, CA, USA)) and pointed to visible discrepancies. We understand that this approach was related to our sorting protocols suggesting standard-sized beads for gate set-up. However, when it was already clear from their initial experiments (shown in the first and second dot plots in the

Figure 2. Flow cytometric concerns regarding VSEL identification and isolation. (**a**) Miyanishi *et al.*[29] described and subsequently isolated large-size, Syto16-bright, CD45$^{-/int}$/Lin$^-$/Sca-1$^+$ murine cells as 'VSEL candidates' (lower left plot, blue box), whereas in our opinion, a visible population of smaller, Syto16-dim cells is the most likely VSEL population (lower left plot, red box). Syto16 is not an optimal DNA dye, and its staining also depends on other intracellular components. It is, for instance, visible in the CD45^{hi}/Lin$^-$/Sca-1$^+$ population (lower right plot, green box), which is almost a half log unit brighter than the majority of FSC^{hi} cells (lower right plot, black box). Moreover, we have a general concern about Sytox-Blue and Sytox16 staining in this study because unfractionated BM cells do not contain any debris or dead cells in such a sample (upper right plot, magenta box), which are visible within subfractions of these cells. (*Dot plots were adopted from a paper by Miyanishi et al. (Stem Cell Reports; 2013, 1, 1–11; part of* Figure 1C*)—published in an open-access journal under Creative Commons Attribution-NonCommercial-No Derivative Works License*).[29] (**b**) Danova-Alt *et al.*,[27] in their recent studies focusing on human VSEL isolation, concluded that CD45$^-$/Lin$^-$/CD133$^+$ cells (which we consider in fact to be a population enriched in human VSELs) as well as CD45$^-$/Lin$^-$/CD34$^+$ cells do not exist in CB, whereas such cells are not only found in our samples (lower dot plots, VSELs marked with blue circles), but also in some CB samples (1, 2 and 3) analyzed by Danova-Alt *et al.* (upper histograms, VSELs marked with blue boxes).[27] These rare stem cell populations were overlooked by Danova-Alt *et al.*[27] because of their visualization on histograms, but are clearly visible when dot plots are used for analysis.[97] (*Histograms were adopted from the paper by Danova-Alt et al. (PLOS One; 2012, 7, e34899; part of* Figure 2B*)—published in an open-access journal under Creative Commons Attribution License*).[27] (**c**) Szade *et al.*[28]in their recent studies on VSELs tried to set up a new protocol for murine VSEL isolation. They were unsuccessful, as they focused on an incorrect fraction and, importantly, lost VSELs during an incorrect gating process.[28] A fraction of the very small objects was lost during: (1) gating on the FSC vs SSC plot (left plot, original gate is shown in black, whereas the suggested classical gate for VSELs is shown by a red circle) and (2) excluding doublets (right-hand plots, original gates are shown in black, whereas suggested gates are shown as red boxes, and arrows indicate areas where VSELs were most likely excluded from sorting gates). (*Dot plots were adopted from the paper by Szade et al. (PLOS One; 2013, 8, e63329; part of* Figure 1A*) published in an open-access journal under Creative Commons Attribution License*).[28] (**d**) Images of selected murine cell populations used for size calculation by imaging cytometry. The left panel shows FlowSight-derived brightfield images of CD45$^{-/int}$/Lin$^-$/Sca-1$^+$/Sytox16$^+$ objects that were isolated as 'VSEL candidates' by Miyanishi *et al.*.[29] Despite the fact that these cells do not represent the VSEL population described by the Ratajczak group, the quality of images is too poor for accurate quantitative analyses, including size calculations.[29] Importantly, images were collected on the instrument with a low sensitivity that may not be useful for VSEL characterization (scale indicates 20 μm). *(Images were adopted from the paper by Miyanishi et al. (Stem Cell Reports; 2013, 1, 1–11; part of Figure S1C)—published in an open-access journal under Creative Commons Attribution-NonCommercial-No Derivative Works License*).[29] The middle panel represents size analysis performed by Szade *et al.*[28] using the ImageStream X system. The histogram shows size analysis of CD45$^-$Lin$^-$Sca-1$^+$c-Kit$^-$ cells, where objects 4-5 μm in size are unfortunately excluded from analysis (as indicated by the orange box), whereas the images are confusingly derived from the distinct CD45$^-$Lin$^-$Sca-1$^+$c-Kit$^+$ cell fraction, which does not represent VSELs. Importantly, the quality of images is too poor for accurate data analysis. *(Data were adopted from the paper by Szade et al. (PLOS One; 2013, 8, e63329; part of Figure S2)—published in an open-access journal under Creative Commons Attribution License*).[28] The right panel shows representative images of murine VSELs (CD45$^-$/Lin$^-$/Sca-1$^+$/7-AAD$^+$ cells), HSCs (CD45$^+$/Lin$^-$/Sca-1$^+$/7-AAD$^+$ cells) and platelets (CD41$^+$/7-AAD$^-$ objects) obtained with the ImageStream 100 system and used for VSEL size analysis.[96] Importantly, with an optimized instrument and good-quality images, even objects as small as 2-μm platelets may be visible and quantified by IDEAS software. Cyan areas show masking used for calculations of cell size by Zuba-Surma *et al.*[96]

original Figure 1)[29] that the cell-size measurement based on the beads employed was not accurate on the particular Aria instrument used for experiments (as all murine white blood cell populations appeared erroneously to be $>10\,\mu m$), the authors continued the size comparison, which was in fact irrelevant to the final sorting outcome. On the other hand, the authors should re-evaluate gating on the instrument they used and VSELs should be sought in the fraction of objects between 4 and 6 μm or even between 4 and 8 μm rather than 4 μm, as all objects are shifted to the upper FSC values when compared with beads (original Figure 1)[29].

Moreover, the authors performed extensive redundant comparisons between instruments,[29] when it is common flow cytometric knowledge that the distribution of both synthetic beads and cells will vary between different instruments (such as, between Aria and MoFlo, or even between machines within one family) and we would strongly expect that the BM cells or other objects would simply 'come back' in different locations on FSC vs SSC plots when compared with the initial instrument employed for sorting of these objects and other instruments.[102] Overall, the authors concluded, based on their flow cytometric size evaluation, that classical flow cytometry is not a reliable method for cell-size analysis and may vary between instruments, with which we certainly agree. Unfortunately, these size comparisons obviously did not bring the authors closer to successful VSEL isolation.

Thus, we would like to highlight that in our daily laboratory practice we employ size-calibrating beads only for the initial experiments when a new instrument/sorter is being used for VSEL isolation, to set up the right protocol or when a major recalibration of the current instrument is performed. There is no 'magic' behind VSEL sorting—just pure biology and the scientifically oriented mind of a good flow cytometry specialist.

Instead of such kind of 'prove-of-proof of principle' experiments, we suggest focusing on a few important technical hints applicable to any type of cell sorter that might be used for VSEL sorting:

- Setting a minimal threshold on the instrument.
- Gating for FSC vs SSC dot plots exclusively on lymphocytes (excluding granulocytes and FSC^{low}/SSC^{hi} objects that may enrich the sorted fraction with different artifacts, apoptotic cells and so on) with extension of the gate to include FSC^{low}/SSC^{low} objects, where a clear cutoff from debris is visible (as shown by our gating strategy in Figure 3).
- Set up a gate enclosing $Lin^-/Sca\text{-}1+$ cells to avoid erythroblast contamination. Apoptotic cell and erythrocyte contamination was reported in the paper by Szade *et al.*,[28] the group that significantly modified sorting and eventually isolated a murine BM cell population distinctly different from VSELs. Moreover, the gating for sorting was performed in an untidy fashion, and very small cells were lost at different steps[28] (Figure 2c). Thus, any major change in gate set-up that results in VSELs being unnecessarily mixed with other objects should be carefully avoided.
- Use dot plots to visualize populations. We generally do not recommend using histograms for any identification and sorting of rare fractions of stem cells, including VSELs. Although in our initial sorting protocols we used histograms visualizing, for example, CD45 expression for final separation of hematopoietic and non-hematopoietic cells,[14,96] we gradually became aware that replacing such a histogram with dot plots (for example, CD45 vs SSC) was much more informative and gave a better gating set-up (Figure 3). Moreover, we found that rare cells such as human VSELs have been simply overlooked by other investigators using histograms showing expression of CD133 and CD34 in $CD45^-/Lin^-$ human UCB samples,[27] although these very rare SCs would be clearly visible in the same samples and in the same analyses if, for instance, CD133 (or CD34) vs SSC dot plots are employed[97] (Figure 2b).
- Importantly, we also exclude any contour plots from our sorting and analysis protocols, which were used by Miyanishi *et al.*,[29] as in our opinion they flatten the view and do not provide complete information about low cell number clusters and the single-cell distribution when we focus on isolation of unique rare objects. We believe that employing contour plots may also potentially lead to technical problems in sorting of rare SCs, as the clear borders of subpopulations are not easily visible.
- Eventually, when gated properly, $CD45^-/Lin^-/Sca\text{-}1^+$ cells will 'come back' as a homogeneous population of small objects in a FSC vs SSC dot plot following back gating (Figure 3).

Importantly, our established protocol for murine VSEL isolation has already been successfully followed and fine-tuned by several laboratories and investigators, including groups in the United States, France, India, Japan and Poland,[16–19,21,22,88,91,103] a fact that was not appreciated by any of the investigators (Miyanishi *et al.*,[29] Danova-Alt *et al.*[27] and Szade *et al.*[28]) who faced problems with VSEL sorting.

We have also noticed the interest by Miyanishi *et al.*[29] in VSEL size measurements by imaging cytometry, a technique in which we are highly skilled and often use in our studies.[94,96,97,104] Unfortunately, in our opinion, FlowSight (Amnis Corporation, Seattle, WA, USA) is not an optimal choice for morphological examination of very small objects such as VSELs. Indeed, the authors should be aware that all of our previous analyses (which they questioned in their paper[29]) were performed with a much more powerful, sophisticated and higher-magnification ImageStream (Amnis Corporation) system.[94,96,97,104] Based on our observations, although FlowSight is capable of analyzing a majority of large cells with its single $\times 20$ objective and fixed optical configuration,[105] it cannot provide images of VSELs of the quality required for reliable image processing, accurate masking and feature calculations by Image Data Exploration and Analysis Software (IDEAS). We have already performed such analyses in our laboratory with a FlowSight demo instrument, and we could not obtain reliable IDEAS data based on FlowSight acquisition, even for human VSELs, which are much larger cells than their murine counterparts (E Zuba-Surma, unpublished data). Therefore, and importantly, the majority of small murine VSELs would not be easily visible on a FlowSight instrument because of its limitations in object resolution. This is also reflected by images in the paper by Miyanishi *et al.*[29] (original Figure S1C[29] and shown as Figure 2d in this article, left panel). FlowSight, with its standard optics pixel resolution of 2 μm, which may be increased maximally to 1 μm/pixel with the Quantitative Imaging Upgrade, still lacks sufficient resolution for accurate VSEL size measurements.[105] Based on our extensive experience in VSEL size evaluation and imaging cytometry, the following facts have become apparent: (1) the only current imaging cytometer that is optically capable of visualizing VSELs is the ImageStream system (both ImageStream 100 and ImageStream X systems, which were used in our VSEL studies), (2) the quality of images of analyzed objects undermines the accuracy of size measurements by IDEAS software for such small objects as VSELs, which is related, for example, to accurate masking set-up by the software, (3) the masking performed by IDEAS should always be verified by the investigator and often fine-tuned (for example, decreased), as the contribution of the cellular halo is much more prominent for small objects than for larger cells, significantly affecting the size calculation when masking automatically based on the brightfield image.[94–97,100,104]

Unfortunately, the data on size measurements by imaging cytometry provided by Miyanishi *et al.*[29] do not in fact challenge our previous results[96] because: (1) these studies were not performed with the high-resolution ImageStream system (our major concern), (2) the quality of the images was poor compared with the VSEL images used for initial calculations by Zuba-Surma

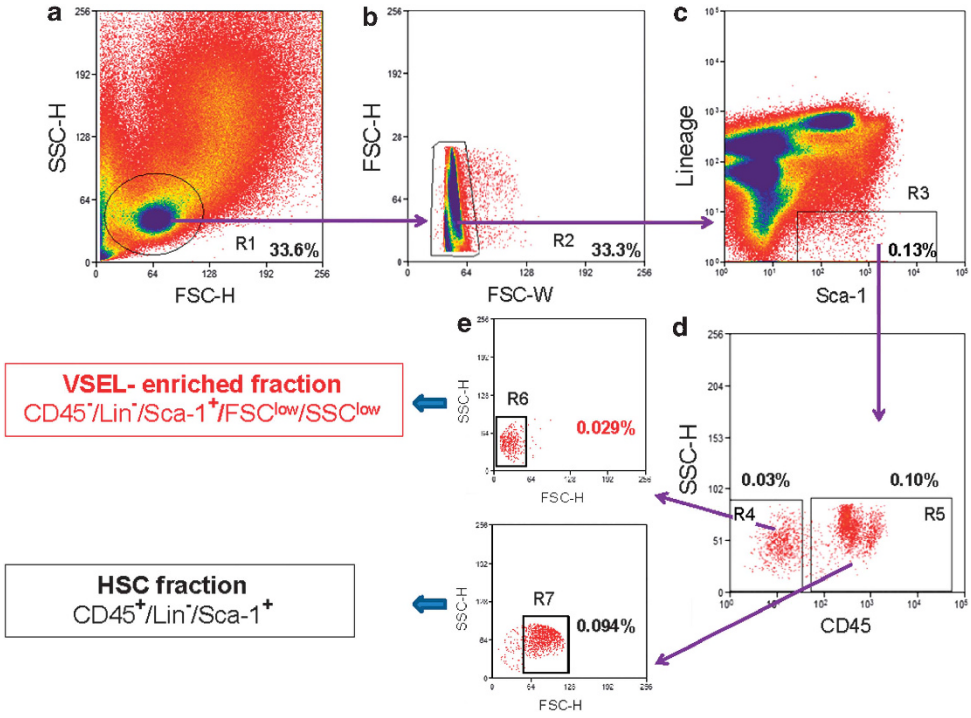


Figure 3. Classical sorting strategy for murine BM-derived VSEL isolation by fluorescence-activated cell sorting (FACS). Agranular, small events ranging from 2 to 10 μm (as initially set up with Flow Cytometry Size beads, Invitrogen/Molecular Probes) are included in an 'extended lymphgate' on an FSC vs SSC dot plot (region R1; **a**). The population of cells from region R1 may be additionally depleted of doublets (gate R2; **b**) to enhance sorting purity (**b**). The single-cell fraction from gate R2 is further analyzed for Sca-1 and Lin expression and exclusively $Lin^-/Sca\text{-}1^+$ cells are gated (R3) to avoid erythroblast contamination (**c**). The population from region R3 is subsequently separated into $CD45^-$ and $CD45^+$ subpopulations visualized in regions R4 and R5, respectively, on a CD45 vs SSC dot plot (**d**). CD45-dim objects are preferentially included in the $CD45^+$ population (region R5). If BM cells are gated strictly according to these steps (with special caution for R1, R3, R4 and R5 gate set-up), the populations of VSELs and HSCs derived from regions R4 and R5, respectively, separate cleanly when 'back-gated' on FSC vs SSC dot plots (**e**). Both stem cell fractions may be additionally purified by final gating, including size-related regions R6 and R7 for VSEL and HSC sorting, respectively. Percentages represent the content of each fraction in the representative murine BM sample.

et al.[96] (Figure 2d) (major concern), (3) the $CD45^{-/int}/Lin^-/Sca\text{-}1^+/Sytox16^{bri}$ larger-cell population was analyzed excluding the $Sytox16^{dim}$ fraction that resembled 'VSEL candidates' (major concern), (4) no information was provided about the analytical approach, including the type of masking or the features used for size calculation, (5) a small number of objects was analyzed and (6) the preparation of cells before analysis was different than in our previously reported studies.[29]

Moreover, it would be very valuable to see all-channel images of the only five small objects included in the analysis (original Figure 2D in Miyanishi *et al.*[29]), which could give further ideas about morphology and the potential similarity of these objects to the previously described VSELs. On the other hand, the authors showed only highly contrasted brightfield images of analyzed cells.[29] Most importantly, in our opinion, an incorrect population was included in the analysis, and it would be much more valuable to investigate $CD45^{-/int}/Lin^-/Sca\text{-}1^+/Sytox16^{dim}$ cells based on their full images, including all stained marker populations, to evaluate both size and potential cell content for assessing the VSEL presence in this fraction. Unfortunately, such an analysis will be reliable only when performed on the more capable ImageStream system.

However, even access to the high-quality ImageStream X system does not guarantee good-quality images that would provide accurate cell-size measurements, as was evident in the study by Szade *et al.*,[28] who failed to isolate VSELs. These authors contradicted our reported results, whereas, in fact, the image quality of objects used for their size measurements was very poor (Figure S2 in Szade *et al.*[28] and Figure 2d in this article, middle panel). Furthermore, the population they focused on as VSELs ($CD45^-/Lin^-/Sca\text{-}1^+/c\text{-}kit^+$) was not comparable to our reported cells. On the other hand, the authors carelessly excluded potentially crucial objects (4-5 μm in size based on their unknown masking and diameter measurements) from size analysis in the $CD45^-/Lin^-/Sca\text{-}1^+/c\text{-}kit^-$ fraction, which could contain VSELs (Figure S2 in Szade *et al.*[28] and Figure 2d in this article, middle panel). Thus, such analysis, even performed on an ImageStream X system, was not professionally performed, which underscores the importance of a skilled and knowledgeable flow cytometry-skilled operator who can fully understand and interpret data generated by this state-of-the-art machine.

Importantly, the paper by Zuba-Surma *et al.*[96] represents one of the first studies in the stem cell research field that employed imaging cytometry for identification and morphological characterization of rare and small-sized stem cell objects, and all calculations were performed in close collaboration with a scientific representative of Amnis Corp. to ensure correct object identification and data accuracy (as shown in example images used in this study and shown in Figure 2d, right panel). Moreover, it was the first paper that clearly showed that the fraction of $FSC^{low}/SSC^{low}/CD45^-/Lin^-/Sca\text{-}1^+$ cells sorted by classical flow cytometry (fluorescence-activated cell sorting) may not only contain a great amount of debris and artifacts, but also include small cellular objects such as VSELs.[96] We further showed a similar phenomenon, with even more debris because of enzymatic digestion, for isolation of VSELs from solid tissues.[95] It is noteworthy that all of our previous VSEL size measurements were performed with a $\times 40$ objective and a resolution of 0.5 μm per pixel (on ImageStream 100 and ImageStream X systems, Figure 2d, right panel).

CONCLUSIONS

Murine BM-derived VSELs are very well described at the morphological and molecular levels and express several morphological and molecular marks that support their pluripotent character. In addition, evidence has accumulated that these cells cross germ layers in their differentiation potential. The fact that they do not form teratomas and do not complete blastocyst development is well explained by changes in expression of certain paternally imprinted genes. Is it really essential that a PSC should show these properties? There should be scientific deliberation on this—in fact this inability to form teratoma may prove to be a very positive feature; such cells might be destined for regenerative medicine. On the other hand, we have always emphasized that, in contrast to their murine counterparts, much work still needs to be done to better characterize human VSELs. Finally, we frankly wonder why, faced with the difficulties of characterizing VSELs by a handful of other groups,[27–29] these groups have not openly, as is usual in science, requested our help. The scientists are wholeheartedly welcome to address themselves to us.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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DISCLOSURE

The University of Louisville is the owner of VSEL patent and some areas of VSEL technology are licensed to Neostem Inc., New York. None of the authors have any stocks in Neostem or other biotechnological stem cell companies.

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