Mini-Review

Very Small Embryonic/Epiblast-Like Stem Cells

A Missing Link To Support the Germ Line Hypothesis of Cancer Development?

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The morphology of several tumors mimics developmentally early tissues, and tumors often express early developmental markers characteristic of the germ line lineage. The presence of these markers in neoplastic cells could reflect the dedifferentiation of somatic cells in which cancer develops or cancer origination in primitive stem cells closely related to the epiblast/germ line. The identification of primitive germ line-derived very small embryonic/epiblast-like stem cells, which are deposited early in embryogenesis in developing organs and persist in several organs into adulthood, raised the possibility that cancer may originate in these cells. In this review, we hypothesize that very small embryonic/epiblast-like stem cells could be a missing link that support the more than 100-year-old concepts of the embryonic rest or germ line origin hypotheses of cancer development; however, further experimental evidence is needed to support this hypothesis. (*Am J Pathol* 2009, 174:1985–1992; DOI: 10.2353/ajpath.2009.081143)

There are several mechanisms leading to cancer development, but it is presently unclear whether cancer originates in differentiated somatic cells or in the stem/progenitor cell compartment. The stem cell origin of cancer hypothesis is based on the assumption that self-renewing stem cells residing in organs and tissues, and not mature differentiated somatic cells such as those lining, for example, the bronchial or stomach mucosa,^{1,2} may acquire and accumulate mutations during a lifetime. These mutations are subsequently maintained in stem cell compartments and self-renewing stem cells may be subjected to additional mutations and epigenetic changes

such that the genome is destabilized and uncontrolled neoplastic proliferation is initiated. Indeed, recent evidence suggests that malignancy arises from accumulation of mutations and maturation arrest of normal stem/progenitor cells rather than by the dedifferentiation of already differentiated cells.^{1,3–6} Accordingly, normal stem cells may acquire mutations and give rise to cancer stem cells, which are subsequently responsible for tumor growth, tumor regrowth after unsuccessful radio-chemotherapy, and establishing distant metastases.

Organ/tissue regeneration and cancer development are likely closely related processes.⁷ Carcinogenesis is very often a response to chronic irritation, inflammation, and tissue damage, potentially developing through misappropriation of homeostatic mechanisms that govern tissue repair and stem cell self-renewal.⁸ Indeed, cancer incidence increases when associated with chronic injury. These observations strongly support a continuous state of repair in the development of cancer, which suggests a role for stem cells in cancer origination.⁹

In addition to these hypothetical considerations, recent research from several laboratories has provided direct evidence that several neoplasms (eg, brain tumors, prostate cancer, melanomas, colon and lung cancer) may in fact originate in the stem cell compartment.^{1,4,10,11} Accordingly, rare populations of primitive stem cells were identified that are able to give rise to tumors in immunodeficient mice that morphologically resemble those tumors from which they were initially purified.¹²

The overall concept that adult tissues contain developmentally primitive cells that can lead to tumors is not

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novel. During the 19th and early 20th centuries, several investigators proposed that cancer may develop in populations of cells that are left in a dormant state in developing organs during embryogenesis. This so-called "embryonic rest hypothesis of cancer origin" was initially postulated by Recamier (1829), Remak (1854), and Virchow (1858). This theory was later elaborated by Durante (1874) and Cohnheim (1875), who suggested that adult tissues may contain embryonic remnants that normally lie dormant, but that can be "activated" to become cancerous. In agreement with those theories, Wright (1910) proposed a germinal cell origin of Wilm's tumor (nephroblastoma) and Beard (1911) postulated that tumors arise from displaced and activated trophoblasts or displaced germ cells. The putative cells responsible for those effects, however, were neither clearly identified nor purified from the adult tissues. Furthermore, since the term "stem cell" was not used at that time in scientific language, it is not clear specifically to which type of cells these early pathologists were referring.

In this review, we present evidence regarding: 1) the existence of a developmentally primitive population of so-called very small embryonic/epiblast-like stem cells (VSELS) in adult tissues that are deposited in early devel-

oping tissues during organogenesis; 2) their relationship to the germ lineage, and 3) some crucial mechanisms that may control/prevent their "unleashed" proliferation. Based on the presence of VSELS in adult tissues, we present our working hypothesis that VSELS could be the missing link that reconciles past theories of the embryonic rest hypothesis of cancer origin with current theories maintaining cancer as a stem cell disorder. The hypothesis presented in this review, however, needs further experimental support and in present form represents a basis for future experimentation. Our goal is to encourage other colleagues to consider the possibility that VSELS could be involved in carcinogenesis.

The Germ Line as Origin and "Skeleton" of the Stem Cell System in the Adult Body

From the developmental point of view, in mammals the germ lineage is "immortal." This immortal cell line passes genomic and mitochondrial DNA to the next generation and during embryogenesis creates "mortal soma" that help the germ line to fulfill its reproductive mission (Figure 1A). The most primitive cell in the germ line is the zygote, a result of fusion of two gametes (germ cells), ie, the

Figure 1. A: Hypothesis of developmental deposition of Oct-4⁺ epiblast/germ line-derived embryonic stem cells (VSELS) in adult tissues. From the developmental and evolutionary point of view, the germ line (red) carries the genome (nuclear and mitochondrial DNA) from one generation to the next. All somatic cell lines bud out (gray) from the germ line during ontogenesis to help germ cells accomplish this mission effectively. Germ potential is established in the fertilized oocyte (zygote) and subsequently retained in the morula, the inner cell mass of the blastocyst, EpiSCs, and PGCs, which directly give rise to gametes, ie, the oocytes and sperm. The first cells that bud out from the germ lineage are trophoectodermal cells that will give rise to the placenta. Subsequently, during gastrulation, EpiSCs are a source of PSCs for all three germ layers (meso-, ecto-, and endoderm) and PGCs. We hypothesize that at this stage, some EpiSCs could be deposited as Oct-4⁺ PSCs in peripheral tissues/organs (red circles). Similarly, some migrating PGCs could stray from their major migratory route and deposit in the genital ridges. Furthermore, it is also possible that, similarly to PGCs, other EpiSCs deposited in developing tissues lose their genomic imprinting (arrows), which can protect the developing organism from the possibility of teratoma formation. However, genomic imprinting erasure will affect some of the aspects of the pluripotentiality of these cells (eg, potential of these cells to contribute to blastocyst development or form teratomas *in vivo*). **B:** Expression of C/T antigens by VSELS. Expression of selected C/T antigens encoded on X chromosome (MageB3, Ssbx2) and on non-X chromosome (Boris) was evaluated in highly purified murine BM-derived VSELS, hematopoietic stem cells, BM mononuclear cells, and an established murine embryonic stem cell line (ESC-D3) by using real-time reverse transcription-polymerase chain reaction, using an ABI Prism 7000 sequence detection system. The relative expression level was calculated by the 2^{-ΔΔCt} method, using β2-microglobulin as an endogenous housekeeping gene and mononuclear cells as a calibrator. Fold differences are shown as means ± SD from at least three independent experiments.

oocyte and sperm, during the process of fertilization. Germ line potential is subsequently maintained in blastomeres of morula and in the inner cell mass of the blastocyst. At the level of the blastocyst, however, a portion of the cells surrounding the blastula “buds out” from the germ line lineage and differentiates toward the trophoblast, giving rise to the placenta. After implantation of the blastocyst in the uterus, germ line potential is maintained in the epiblast, which contains small PSCs and ultimately gives rise to the precursors of gametes and all somatic tissues. Thus, PSCs from the epiblast (epiblast-derived stem cells or EpiSCs) become stem cells that maintain germ potential in the adult body (primordial germ cells or PGCs), multipotent stem cells for meso-, ecto-, and endoderm in gastrula, and unipotent tissue-committed stem cells (TCSCs) for all of the developing organs and tissues in the embryo proper. We hypothesize that some of those EpiSCs^{13,14} could survive as Oct-4⁺ pluripotent VSELs^{19,21} among TCSCs in peripheral tissues/organs (Figure 1A).

Identification in Adult Tissues of VSELs That Express Several Early Developmental Markers

Stem cells committed to one tissue have been postulated to trans-dedifferentiate into stem cells for other organs and tissues (eg, hematopoietic stem cells could “become” cardiac stem cells). A few years ago, we proposed an alternative explanation to stem cell plasticity or stem cell trans-dedifferentiation—that some of the positive “plasticity data” could be explained by the presence in adult tissues of pluripotent stem cells (PSCs). We hypothesized that adult tissues contain rare PSCs that could co-exist with TCSCs.¹⁵ Therefore, bone marrow (BM)-derived hematopoietic stem cells used in several plasticity experiments could be “contaminated” by primitive PSCs, which were actually responsible for the tissue regeneration.

To test this hypothesis, we began a search for putative PSC candidates. According to the definition of pluripotentiality, PSCs would be able to differentiate into cells from all three germ layers (ecto-, meso-, and endoderm). We succeeded in isolating such cells from adult murine BM and then from several other murine organs.¹⁶ Based on morphological criteria and expression of early developmental markers, we named these cells “very small embryonic like stem cells” or VSELs. Subsequently, a similar/corresponding population of stem cells was identified in human umbilical cord blood.¹⁷ Our recent research indicates that these cells share several developmental markers of early epiblast stem cells; therefore, perhaps a more correct name for these cells would be “very small embryonic/epiblast-like stem cells.” Thus we will employ this term in the current review.

To isolate pluripotent VSELs from murine adult BM, we assumed from our preliminary experimental data that these cells would be CD133⁺CXCR4⁺ both in mouse and humans and Sca-1⁺ in mice. In both species, they were

expected to be negative for the pan-hematopoietic marker CD45 (CD45[−]).¹⁸ Our chemotactic experiments, in which we analyzed BM cells collected from the transwell lower chambers after CXCR4 receptor ligand (α -chemokine stromal derived factor-1, SDF-1) chemotaxis assays, revealed that murine BM contains some very rare and small CXCR4⁺ cells (3–5 μ m in diameter). To our surprise, these small CXCR4⁺ cells expressed early developmental markers characteristic of PSCs. Based on these data, we added small size to the crucial sorting criterion used to purify these putative PSCs from the BM.^{19,20}

To sort small pluripotent VSELs (<6 μ m, Sca-1⁺CXCR4⁺lin[−]CD45[−] cells) from murine BM by fluorescence-activated cell sorting, we used a novel size-based approach using size bead markers to extend the lymphocyte gate to include the so-called small event region (2–10 μ m). This region mostly contains cell debris, large platelets, and erythrocytes; however, it also includes some rare nucleated cell events. Most sorting protocols exclude events smaller than 6 μ m in diameter to remove cell debris, erythrocytes, and platelets, so small VSELs are usually excluded from the sorted cell populations, which explains why these small cells were not purified previously. It is likely that similarly small cells are present in preimplantation blastocysts in so-called primitive embryonic ectoderm or epiblast, which ultimately gives rise to all three germ layers and the embryo proper.

By using flow cytometry supported by size marker beads and transmission electron microscopy, we demonstrated that these small (<6 μ m) cells constituted the majority of Sca-1⁺CXCR4⁺lin[−]CD45[−] cells isolated from adult murine BM. By transmission electron microscopy, sorted VSELs also displayed a very primitive morphology. They contained large nuclei surrounded by a tiny rim of cytoplasm enriched for mitochondria. On the surface, they expressed high levels of stage-specific embryonic antigen-1 (SSEA-1) in mice and SSEA-4 in humans.^{17,19} They also expressed early embryonic transcription factors such as Oct-4, Nanog, Klf-4, and Rex-1, both at the mRNA and protein levels. Oct-4 and Nanog promoters were recently confirmed by our group to have an open-type chromatin structure, confirming expression.²¹

In addition to Oct-4, SSEA-1, and CXCR4, VSELs also express several other markers of epiblast/germ line cells: fetal-type alkaline phosphatase, Mvh, Stella, Fragilis, Nobox, Hdac6. VSELs have high telomerase activity and, similarly to embryonic stem cells, do not express major histocompatibility class I and human leukocyte antigen-D-related antigens. They are negative for typical mesenchymal stem cell markers such as CD90[−]CD105[−]CD29[−]²¹; however, this does not preclude that, as a population of PSCs, they may ultimately give rise to mesenchymal stem cells as well.

Using ImageStream system analysis, which allows for statistical analysis of various cellular parameters acquired by fluorescence-activated cell sorting in suspension during flow analysis as well as direct visualization of cells via high-resolution bright field, dark field, and fluorescence images (as small as 1 μ m in diameter), we confirmed with greater precision that VSELs are ~3.6 μ m

in diameter. We also noticed that VSELs have a significantly higher ($P \leq 0.05$) nuclear/cytoplasmic ratio as compared with hematopoietic stem cells: 1.47 ± 0.17 and 0.82 ± 0.03 , respectively. Furthermore, VSELs had a significantly lower ($P \leq 0.05$) cytoplasmic area as compared with hematopoietic stem cells (5.41 ± 0.58 and 33.78 ± 1.68 , respectively).²⁰

By using flow cytometry, ImageStream system analysis, confocal microscopy, and real-time reverse transcription-polymerase chain reaction, similar cells could be also identified and isolated from several other organs in adult mice.²⁰ The highest total numbers of Oct-4⁺ VSELs were found in the brain, kidneys, muscles, pancreas, and BM. These observations support our hypothesis that a population of very primitive cells expressing germ line/epiblast markers (Oct-4, SSEA-1) is deposited early during embryogenesis in various organs and survives into adulthood. However, further studies are needed to determine whether these cells, after being isolated from various adult human organs similarly to their murine BM-derived counterparts, are endowed with PSCs properties and, more importantly, whether similar cells are also present in human organs. The presence of similar cells in human umbilical cord blood and mobilized peripheral blood lends support to this possibility.¹⁷

VSELs Have Functional Characteristics of PSCs

Despite their small size, VSELs possess diploid DNA. Interestingly, approximately 5 to 10% of purified VSELs, if plated over a C2C12 murine myoblastic cell line feeder layer, are able to form spheres that resemble embryoid bodies. Cells from these VSEL-derived spheres (VSEL-DSs) are composed of immature cells with large nuclei containing euchromatin and are CXCR4⁺SSEA-1⁺Oct-4⁺, just like freshly purified VSELs. Furthermore, cells isolated from these spheres are able to differentiate in appropriate culture conditions into cells belonging to all three germ layers, ie, cardiomyocytes (mesoderm), neural cells (ectoderm), and insulin-producing cells (endoderm). This provides *in vitro* evidence that VSELs are in fact PSCs.²¹

Similar VSEL-DSs were also formed by VSELs isolated from murine fetal liver, spleen, and thymus. Interestingly, formation of VSEL-DSs was associated with a young age in mice and no VSEL-DSs were observed in cells isolated from older mice (>2 years). This age-dependent content of VSELs in BM may somehow explain why the regeneration processes is more efficient in younger individuals.²² We also noticed differences in the content of these cells among BM mononuclear cells between long- and short-lived mouse strains.¹⁸ The concentration of these cells is much higher in the BM of long-lived (eg, C57Bl6) as compared with short-lived (DBA/2J) mice.¹⁸ This observation leads to the potential exciting task of identifying genes responsible for tissue distribution and expansion of these cells, as these genes could be involved in controlling the life span of mammals.

VSELs are also highly mobile and respond robustly to a SDF-1 gradient, adhere to fibronectin and fibrinogen, and may interact with BM-derived stromal fibroblasts. Confocal microscopy and time-lapse studies revealed that these cells attach rapidly to, migrate beneath, and reside inside invaginations/cell membrane pockets (emperipolesis) of marrow-derived fibroblasts.¹⁸ Fibroblasts secrete SDF-1 and other chemoattractants, which may create a homing environment for small CXCR4⁺ VSELs. This robust interaction of VSELs with BM-derived fibroblasts has an important implication. It is possible that isolated BM and other tissue fibroblastic cells (eg, mesenchymal stem cell,²³ multipotent adult stem cells,²⁴ multipotent adult progenitor cells,²⁵ or marrow-isolated adult multilineage inducible cells²⁶) may be contaminated to some degree by these tiny cells that could contribute to "plasticity" of marrow-derived adherent cell populations (eg, mesenchymal stem cells or multipotent adult stem cells).

Erasure of Genomic Imprinting: The Mechanism That Prevents Teratoma Formation from Primitive Germ Line Cells

As mentioned above, the epiblast becomes a source for all types of TCSCs. Accordingly, at 7.25 days after conception in mice, a portion of pluripotent EpiSCs is specified to a population of PGCs²⁷ that will migrate to the genital ridges and form oocytes or sperm during gametogenesis.²⁸ Shortly after PGC specification, the remaining EpiSCs, which we envision to be related to the germ line lineage (Figure 1A), give rise to multipotent/unipotent stem cells for developing germ layers in gastrula and, subsequently, TCSCs for tissues and organs. However, we postulate that these primitive epiblast/germ line PSCs are not completely eliminated from the developing organism by the differentiation process and some of them may survive among their progeny, as with tissue TCSCs. The proliferative potential of these developmentally primitive PSCs (eg, VSELs?) has to be very well controlled to prevent the possibility of teratoma formation.²¹ Such mechanisms that keep the most primitive cells in developing organisms under control were initially very well described for PGCs. This dynamic is known as "erasure of the genomic imprinting."^{29,30}

From a developmental point of view, PGCs are the most important population of stem cells because, as precursors of gametes, they are directly responsible for passing genetic information to the next generation. However and somehow surprisingly, if these developmentally early cells are isolated from the developing embryo after 11 days post-conception (when they migrate to the genital ridges) and are cultured *ex vivo*, they rapidly undergo terminal differentiation or apoptosis.³⁰ Interestingly, they also do not complement blastocyst development, are not able to provide fully functional nuclei during nuclear transfer in the process of clonote formation, and do not grow teratomas after inoculation into immunodeficient mice. Therefore, PGCs obviously lack all of the currently

approved criteria of pluripotentiality. These data suggest that PGCs are protected from uncontrolled proliferation/expansion by regulatory mechanism(s).

The lack of PGC pluripotentiality results from epigenetic modification and erasure of the genomic imprinting on differentially methylated regions (DMRs) of imprinted genes.³¹ The murine genome contains approximately 80 genes that show differential DNA methylation patterns either on maternal or paternal chromosomes (eg, H19, Igf2, Igf2R, Snrpn, KCNQ1, and Rasgrf1).^{32,33} As a result of imprinting, Igf2 is expressed from the paternal and H19 is expressed from the maternal chromosome. Proper imprinting of these two important genes maintains their balanced expression: H19 encodes cell proliferation repressive mRNA and Igf2 encodes an important growth factor. DNA methylation erasure on DMR for Igf2-H19 leads to overexpression of negative regulatory H19 mRNA and down-regulation of Igf2. The change in expression of these genes contributes to PGC quiescent.

Indeed, re-establishment of proper imprinting on the DMR for the Igf2-H19 locus on one set of chromosomes was essential for creating parthenogenic mice derived from two female haploid sets of DNA.³⁴ Furthermore, loss of imprinting on the DMR for the Igf2-H19 locus is observed in Beckwith-Wiedemann syndrome, which is connected with the frequent incidence of organomegaly in several pediatric sarcomas (eg, rhabdomyosarcomas, nephroblastomas).^{35,36} As a result of genomic imprinting erasure, affected cells highly express Igf2, which may act as an autocrine growth factor and down-regulate the cell cycle negative regulatory H19 mRNA, resulting in uncontrolled proliferation and expansion.

Overall the process of erasure of the genomic imprinting occurs very early during gastrulation at a time when the PGCs begin to migrate to the genital ridges.²⁷ This process is one of the basic mechanisms preventing uncontrolled proliferation, initiation of potential parthenogenesis, and potential teratoma formation by PGCs. Conversely, proper genomic imprinting is required for PSCs to be able to complement blastocyst development, provide nuclei for clonote formation after nuclear transfer, and grow teratomas in immunodeficient mice.³⁷ Since migrating PGCs erase this imprinting, they are not able to display these "gold standard" pluripotency criteria in appropriate experimental models.

However, if plated over murine fetal fibroblasts in the presence of selected growth factors (leukemia inhibitory factor, basic fibroblast growth factor, and kit ligand), PGCs may undergo epigenetic modifications forced by *in vitro* culture conditions and regain genomic imprinting.³⁸⁻⁴⁰ Re-establishing imprinting in PGCs endows them again with "immortality." This immortalized population of PGCs known as embryonic germ cells is equivalent to embryonic stem cells in many aspects. For example, similar to embryonic stem cells, embryonic germ cells contribute to all three germ layers, including the germ cell lineage, after injection into a blastocyst (blastocyst complementation assay), provide functional nuclei for clonote after nuclear transfer, and form teratomas after injection into living mice. Thus, it is evident that

proper genomic imprinting is vital for cells from the germ line to retain *in vivo* pluripotentiality.

We postulated that genomic imprinting erasure of some developmentally crucial genes could be involved in controlling the quiescent status of epiblast/germ line-derived VSELs.²¹ Similarly to PGCs, this process could be potentially reversible. In fact, recent research from our group demonstrated a unique molecular signature of genomic imprinting on selected developmentally imprinted genes that partially mimics those observed in PGCs (eg, erasure of DMR imprint for Igf2-H19 locus).²¹ Therefore, the erasure of methylation on some DMRs in VSELs may explain the quiescent status of these very primitive stem cells. Furthermore, we envision that the methylation status of these crucial DMRs could be manipulated in VSELs and potentially reverted. By re-establishing proper imprinting, VSELs could become fully pluripotent in appropriate *in vivo* tests (eg, blastocyst complementation, teratoma formation).

Data Supporting a Theory of Cancer Origin in the Germ Cell Compartment: A Potential VSEL Link?

There are several indications that cancer originates in cells closely related to the germ line. Incidence of "classical" germ line tumors such as seminoma, ovarian tumor, yolk sac tumor, mediastinal and brain germ cell tumors, teratoma, and teratocarcinoma could be attributed to migrating PGCs deposited in adult tissues that have gone astray during developmental migration en route to the genital ridges.⁴¹⁻⁴³ In addition, cancer testis (C/T) antigens (approximately 40 identified so far), which are encoded by genes normally expressed in the germ line only, are also expressed in various tumor cell types (eg, gastric, lung, liver, bladder carcinomas, melanomas, medulloblastomas, pediatric sarcomas, germinal tumors).^{44,45} This indicates cancer may originate in germ line cells; however, the expression of these genes in cancer cells could be alternatively explained as a re-expression of the primitive germ line potential in mutated/epigenetically changed cancer cells. Of note, we observed that VSELs express several C/T antigens (Figure 1B). As such, they could potentially be a population of stem cells that give rise to all C/T antigen-expressing cells.

It is also well known that several types of cancer secrete either the β subunit of human chorionic gonadotropin⁴⁶ or its fragments and/or carcinoembryonic antigen. These germ line markers could indicate the germ line origin of tumors or alternatively, as in the case of C/T antigens, a re-expression of the primitive germ line potential in mutated cells. Finally, Oct-4 transcription factors, which are a marker of embryonic, epiblast, and germ line PSCs, are also expressed in various tumor types (eg, gastric, lung, bladder, oral mucosa carcinomas, germinal tumors).⁴⁷ However, the true expression of Oct-4 in cancer cells must be considered first after excluding the possible expression of Oct-4 pseudogenes.

Table 1. Potential Contribution of VSELs in the Development of Malignancies

Tumor types	Potential mechanisms
Teratomas, teratocarcinomas	Persistent genomic imprinting in Oct-4 ⁺ cells (epiblast-derived?), additional mutations
Germinomas, seminomas, teratomas, dermoid cyst, hydatidiform mole	Cells left along PGC migratory route, persistent genomic imprinting, additional mutations?
Pediatric sarcomas (rhabdomyosarcoma, neuroblastoma, Ewing's sarcoma, nephroblastoma, Wilm's tumor)	Mutated Oct-4 ⁺ cells in various peripheral tissue locations?
Other malignancies (eg, <i>Helicobacter pylori</i> -related stomach cancer, smoke-related lung cancer, colon cancer)	Circulating Oct-4 ⁺ cells incorporated at the wrong time to the wrong place, additional mutations? Cell fusion leading to chromosomal instability?

VSELs highly express Oct-4 at the mRNA and protein level. The true expression of Oct-4 in these cells was confirmed using sequence specific primers and, more importantly, by showing that the Oct-4 promoter DNA in VSELs is in a hypomethylated state and thus is transcriptionally active.²¹

Are VSELs the Missing Link That Reconciles the Embryonic Rest/Germ Line Origin of Cancer and the Stem Cell Theory of Cancer Development?

We assert that the Oct-4⁺ VSELs recently identified in adult tissues may somehow unify and fully support the cancer stem cell origin theories (Table 1). First, we envision that if the genomic imprinting in VSELs is not erased, they may retain postdevelopmental *in vivo* pluripotency

and grow teratomas and teratocarcinomas. Second, if PGCs, which are closely related to VSELs, migrate astray from the major migratory route to the genital ridges and ultimately settle down in various organs, they may give rise to germinal tumors. Third, if VSELs acquire some critical epigenetic changes (eg, acquire hypermethylation on DMR for the Igf2-H19 locus), they may develop into the many types of pediatric sarcomas (eg, rhabdomyosarcoma, neuroblastoma, Ewing's sarcoma, Wilm's tumor). In support of this, we observed a strong correlation between the number of these Oct-4⁺ cells that persist in postnatal tissues and the coincidence of these types of tumors in pediatric patients.⁴⁸ Finally, it is possible that if VSELs are mobilized at the wrong time into peripheral blood and deposited in areas of chronic inflammation instead of playing a role in regeneration, they may contribute to the development of other malignancies (eg, stomach cancer or lung cancer). To support this

Figure 2. A: Potential involvement of VSELs in carcinogenesis. first scenario: VSELs may give rise to cancer (stem) cells (dark irregular cell) if their genomic imprinting is not erased or the VSEL acquires mutations and, as a result of this, gives rise to teratomas/teratocarcinomas or sarcomas (eg, rhabdomyosarcoma, neuroblastoma, nephroblastoma), respectively. This first scenario could play a significant role in pathogenesis of pediatric tumors. In the second scenario, VSELs may fuse due to the chronic inflammation/fusogenic agent within a normal somatic cell (white box with dark nucleus) and give rise to a heterokaryon (blue cells with nucleus). The tetraploid heterokaryon undergoes subsequent selection and gives rise to the aneuploid cancer (stem) cell. This scenario could play a more important role in pathogenesis of tumors developing in adult patients. In the third scenario, VSELs may indirectly contribute to tumorigenesis by providing vasculature and stroma cells for growing tumor tissue. This may occur in tumors developing both in young and older patients. **B:** Increase of circulating VSELs in PB of mice inoculated with murine myoblastic C2C12 cells. C2C12 cells were injected into the anterior tibialis muscle of C57BL/6 mice. Mice (treated and control groups) were sacrificed at 8, 16, 24, and 40 days after the onset of injection. Following euthanasia, blood samples (1.0–1.5 ml from each mouse) were collected in heparin-rinsed syringes for the isolation of peripheral blood mononuclear cells. The numbers of VSELs and white blood cells (WBC) were enumerated in PB by fluorescence-activated cell sorting. Data are pooled from four independent experiments.

further, several tumor types may express embryonic markers including Oct-4 and, as reported, BM-derived stem cells that may develop several sarcomas including teratomas in the presence of carcinogens.⁴⁹ It is possible that VSELs hiding among BM-derived mesenchymal stem cells contribute to this process.

Several groups have proposed that cancer is a chromosomal rather than a genetic disease. Accordingly, carcinogenesis could be initiated by aneuploidies induced by oncogenes, which unbalance gene expression and lead to the selection of aneuploid clones of transformed cells.⁵⁰ In this context, VSELs, as highly fusogenic cells, could be potential fusion partners for somatic cells. VSELs could provide several transcripts characteristic for early developmental cells (eg, Oct-4, Klf-4), while somatic cells could supply chromosomes that show proper genomic imprinting. The formation of such heterokaryons could be a first step in the selection of aneuploid immortal cells. Each of these possibilities of how VSELs could hypothetically contribute to cancer initiation and expansion are shown in Figure 2A.

Finally, we also postulate that mobile VSELs could be chemoattracted by the expanding tumor tissue and could potentially be involved in tumor expansion and growth by providing vessels and stroma. In this latter case, since the expanding tumor secretes several chemoattractants for VSELs (eg, SDF-1, hepatocyte growth factor/scatter factor, vascular endothelial growth factor), these cells could wrongly recognize the expanding tumor as regenerating tissue. After being mobilized into the PB, VSELs could be incorporated into the tumor microenvironment to provide vessels and stroma. To support this notion, we noticed an increase in circulating VSELs in the PB of immunodeficient mice inoculated with human tumor cells (Figure 2B).

Conclusion

We recently identified a unique population of VSELs that express several epiblast/germ line markers, thus implying that these rare cells could be the origin of several malignancies. The presence of VSELs in adult tissues may support the embryonic rest or the germ line origin hypotheses of cancer development and reconcile them with currently postulated stem cell theories of cancer development. In fact, markers present in VSELs such as Oct-4, SSEA, or C/T antigens are identified in several malignancies present in pediatric and adult patients. We envision that VSELs can initiate cancer by acquiring mutations, keeping the genomic imprinting, and fusing with other somatic cells as well as contribute to tumor expansion by providing stroma and endothelial precursors. However, the potential involvement of VSELs in tumorigenesis requires further studies.

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