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Adult murine bone marrow-derived very small embryonic-like stem cells differentiate into the hematopoietic lineage after coculture over OP9 stromal cells

Janina Ratajczak[a,b], Marcin Wysoczynski[a], Ewa Zuba-Surma[a,c], Wu Wan[a], Magda Kucia[a], Mervin C. Yoder[d], and Mariusz Z. Ratajczak[a]

[a]*Stem Cell Institute at James Graham Brown Cancer Center, University of Louisville, Louisville, Ky., USA;* [b]*Department of Physiology, Pomeranian Medical University, Szczecin, Poland;* [c]*Department of Medical Biotechnology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland;* [d]*Department of Pediatrics, Indiana University School of Medicine, Indianapolis, Ind., USA*

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Objective. **We recently identified a population of small Sca-1$^+$/Lin$^-$/CD45$^-$ cells in adult murine bone marrow that express several epiblast/germ line and pluripotent stem cell markers (e.g., Oct-4 and SSEA-4) that we named "very small embryonic-like stem cells" (VSELs). In this report, we test the hypothesis that VSELs can differentiate along the hemato/lymphopoietic lineage.**

Materials and Methods. **Purified from bone marrow, VSELs were primed/cocultured over OP9 stroma cell line and subsequently tested in vitro and in vivo assays for their hematopoietic potential. In parallel, cells derived from VSELs were evaluated for expression of hematopoietic genes and surface markers.**

Results. **Although we observed that freshly isolated VSELs do not exhibit in vitro and in vivo hematopoietic potential, they may, after coculture over OP9 stromal cells, differentiate along the hematopoietic lineage in a similar way as embryonic stem cells or inducible pluripotent stem cells. "OP9-primed," VSEL-derived cells acquired expression of several hemato/lymphopoiesis-specific genes and markers, gave rise to hematopoietic colonies in vitro, and protected lethally irradiated mice in both primary and secondary transplant models on transplantation. We also observed that, compared to hematopoietic stem/progenitor cells, VSELs are highly resistant to total body irradiation.**

Conclusions. **Based on these observations, we postulate that VSELs are the most primitive murine bone marrow−residing population of stem cells that have the potential to become specified into the hematopoietic lineage and may share some of the characteristics of long-term repopulating HSCs.** **© 2011 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

In addition to hematopoietic stem/progenitor cells (HSPCs), bone marrow (BM) contains other types of developmentally early stem/progenitor cells, including mesenchymal stem cells and endothelial progenitor cells [1,2]. Cells with the ability to differentiate into multiple germ layers were recently identified in BM tissue, including multipotent adult progenitor cells [3], marrow-isolated adult multipotent-induced cells [4], as well as a population of so-called very small embryonic/epiblast-like stem cells (VSELs) identified by us [5].

We have hypothesized that VSELs are deposited at the beginning of gastrulation in developing tissues and play an important role as a backup population for tissue-committed stem cells [5]. We envision that during steady-state conditions these cells may be involved in tissue rejuvenation and participate in tissue repair after organ injury [6,7]. Molecular analysis of adult BM-derived, purified VSELs revealed that they (1) express pluripotent stem cell (PSC) genes (e.g., Oct-4, Nanog, Klf-4, and SSEA-1), (2) share several markers characteristic of epiblast as well as migratory primordial

Current address: Department of Medical Biotechnology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland.

Offprint requests to: Janina Ratajczak, M.D., Ph.D., Stem Cell Institute at James Graham Brown Cancer Center, University of Louisville, 500 S. Floyd Street, Room 107, Louisville, KY 40202, USA; E-mail: j0rata01@louisville.edu

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germ cells (PGCs), and (3) possess a unique pattern of genomic imprinting (namely erasure of differently methylated regions at the Igf2-H19 and Rasgrf1 loci and hypermethylation at the KCNQ1 and Igf2R loci) [5–7]. These characteristics suggest that VSELs are related to epiblast-derived migrating PGC-like cells and, despite their PSC character, changes in the epigenetic signature of imprinted genes keep these cells quiescent in adult tissues and prevent them from uncontrolled proliferation (e.g., teratoma formation). On the other hand, epigenetic changes or mutations that lead to activation of imprinted genes in these cells could potentially lead to tumorogenesis [8]. Using appropriate experimental models, we have already demonstrated that VSELs may give rise to mesenchymal stem cells [9], neural cells, cardiomyocytes, and insulin-producing cells [4].

It is well known that murine and human BM contain a population of long-term repopulating hematopoietic stem cells (LT-HSCs); however, the exact phenotype of these cells is not well defined and varies between reports [10]. For example, based on surface expression markers, the phenotype of murine long-term engrafting LT-HSCs is described as Thy 1.1^{lo} Lin^- Sca^{high} $Mac1^-$ $CD4^-$ or $Thy1.1^{low}$ $Flk\text{-}2^-$ [11] or $CD150^{high}$ $CD34^{-/low}$ $c\text{-}Kit^+$ $Sca\text{-}1^+$ Lin^- [12]. However, evidence is accumulating that these surface markers do not identify all the cells that reside in BM and are endowed with long-term hematopoietic potential [13–15].

Because of this problem, we decided to evaluate whether BM-residing, developmentally early VSELs isolated by our team can give rise to the hemato/lymphopoietic lineage. When employing in vitro and in vivo assays in cocultures with OP9 stromal cells, we observed that VSELs may become specified into the hemato/lymphopoietic lineage. Thus, our data suggest that VSELs may share some characteristics with the most primitive LT-HSCs.

Materials and methods

Isolation and fluorescence-activated cell sorting (FACS) of VSELs from murine BM

This study was performed in accordance with the guidelines of the Animal Care and Use Committee of the University of Louisville School of Medicine and with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, publication no. NIH 86-23).

VSELs were isolated from BM of adult male or female transgenic (with enhanced green fluorescence protein) C57BL/6 mice (4–8 weeks old; Jackson Laboratory, Bar Harbor, ME, USA). Briefly, BM was flushed from tibias and femurs and the population of total nucleated cells (TNCs) was obtained after lysis of red blood cells using 1× BD Pharm Lyse Buffer (BD Pharmingen, San Jose, CA, USA). TNCs were subsequently stained for CD45, hematopoietic lineage markers (Lineage [Lin]), and Sca-1 antigen for 30 minutes in medium containing 2% fetal bovine serum. The following anti-mouse antibodies (BD Pharmingen) were used for staining: rat anti-CD45 (allophycocyanin-Cy7; clone 30-F11), anti-CD45R/B220 (phycoerythrin [PE]; clone RA3-6B2), anti–Gr-1 (PE; clone RB6-8C5), anti–T-cell receptor-αβ (PE; clone H57-597), anti–T-cell receptor-γδ (PE; clone GL3), anti-CD11b (PE; clone M1/70), anti-Ter119 (PE; clone TER-119), and anti–Ly-6A/E (Sca-1) (biotin; clone E13-161.7, with streptavidin-conjugated to PE-Cy5). Cells were then washed, re-suspended in RPMI 1640 medium with 10% fetal bovine serum, and sorted by MoFlo cell sorter (Dako, Carpinteria, CA, USA). The $Sca\text{-}1^+Lin^-CD45^-$ cells (VSELs) and control $Sca\text{-}1^+Lin^-CD45^+$ cells (HSCs) were isolated according to the gating and sorting strategy described below (Fig. 1).

Ex vivo differentiation of VSELs into hematopoietic cells in primary cocultures over OP9 stromal cells

Freshly sorted $Sca\text{-}1^+Lin^-CD45^-$ VSELs and $Sca\text{-}1^+Lin^-CD45^+$ HSCs from BM were plated over OP9 cells in α–minimum essential medium with 20% fetal bovine serum (Molecular Probes, Invitrogen) for 5 days and subsequently trypsinized, washed by centrifugation in α–minimum essential medium, and replated in methylcellulose-based medium (StemCell Tech, Vancouver, BC, Canada).

Evaluation of the clonogenic potential of sorted cells in methylcellulose cultures

VSELs or HSCs freshly isolated from BM or cells harvested from OP9 cultures (primary cultures) were plated in methylcellulose-based medium (StemCell Tech) supplemented with murine stem cell growth factor, interleukin-3, granulocyte-macrophage colony-stimulating factor, FMS-like tyrosine kinase 3 ligand, thrombopoietin, erythropoietin, and insulin growth factor–2. Cells were cultured for 5 days and the colonies formed were scored. Subsequently, methylcellulose cultures were solubilized and trypsinized and the resulting cells were washed by centrifugation in α–minimum essential medium and plated into secondary methylcellulose cultures. Cells were grown in the presence of the same growth factors and replated after 5 days into new methylcellulose cultures. The same procedure was repeated for the next two passages.

Polymerase chain reaction (PCR) analysis of gene expression in freshly sorted cells and OP9-expanded cells

Total RNA from various cells (approximately 20,000 cells) was isolated using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) and genomic DNA removed using the DNA-free Kit (Applied Biosystems, Foster City, CA, USA). Isolated messenger messenger RNA was reverse-transcribed with Taqman Reverse Transcription Reagents (Applied Biosystems) according to manufacturer's instructions. Reverse transcriptase PCR (RT-PCR) was performed using Amplitaq Gold (Applied Biosystems) with one cycle of 8 minutes at 95°C; 2 cycles of 2 minutes at 95°C, 1 minutes at 62°C, and 1 minute at 72°C; 38 cycles of 30 seconds at 95°C, 1 minute at 62°C, and 1 minute at 72°C; and 1 cycle of 10 minute at 72°C using sequence-specific primers. Quantitative measurement of target transcript expression was performed by real-time quantitative PCR using an ABI Prism 7500 Sequence Detection System (Applied Biosystems). Complementary DNA from indicated cells was amplified using SYBR Green PCR Master Mix (Applied Biosystems) and specific primers. All primers were designed with Primer Express software (Applied Biosystems), with at least one primer in each pair containing an exon-intron boundary. The threshold cycle (*C*t) was determined and relative quantification of the expression level of target genes was obtained with the $2^{-\Delta\Delta Ct}$ method, using β2-microglobulin as an endogenous control gene