

Identification of the Niche and Phenotype of the First Human Hematopoietic Stem Cells

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SUMMARY

In various vertebrate species, the dorsal aorta (Ao) is the site of specification of adult hematopoietic stem cells (HSCs). It has been observed that the upregulation of essential hematopoietic transcription factors and the formation of specific intra-aortic hematopoietic cell clusters occur predominantly in the ventral domain of the Ao (AoV). In the mouse, the first HSCs emerge in the AoV. Here, we demonstrate that in the human embryo the first definitive HSCs also emerge asymmetrically and are localized to the AoV, which thus identifies a functional niche for developing human HSCs. Using magnetic cell separation and xenotransplantations, we show that the first human HSCs are CD34⁺VE-cadherin⁺CD45⁺C-KIT⁺THY-1⁺Endoglin⁺RUNX1⁺CD38^{-/lo}CD45RA⁻. This population harbors practically all committed hematopoietic progenitors and is underrepresented in the dorsal domain of the Ao (AoD) and urogenital ridges (UGRs). The present study provides a foundation for analysis of molecular mechanisms underpinning embryonic specification of human HSCs.

INTRODUCTION

Hematopoietic stem cells (HSCs) emerge early during embryogenesis and maintain multilineage hematopoiesis throughout the entire lifespan of the organism through continuous self-renewal and differentiation (Dzierzak and Speck, 2008; Medvinsky et al., 2011). Although early development of mouse HSCs has been extensively investigated, early human HSC development is poorly understood. A better understanding of this process could be of practical importance. Bone marrow and umbilical cord blood-derived HSC transplantation is performed for a number of therapeutic indications in the clinic, but the availability of suitable donors is inadequate. Many research groups are investigating the possibility of generating HSCs under controlled conditions in the laboratory. Embryonic and induced pluripotent stem cells can differentiate in vitro into the majority of cell types, including various hematopoietic cells, and could be an ideal source of customized HSCs for clinical applications (Kaufman, 2009). However, the generation of true transplantable HSCs remains a significant challenge. A better understanding of the embryonic development of human HSCs may be instrumental for developing novel protocols for their generation in vitro.

Due to the poor availability of human embryonic tissues and the limitations of xenotransplantation models, studies on early human hematopoietic development were largely based on immunohistological and in vitro techniques, which did not encompass HSCs (Huyhn et al., 1995; Oberlin et al., 2002; Tavian et al., 2001). The development of

severely immunodeficient NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/Sz (NSG) recipient mice that are highly receptive for human cells has enhanced the capacity of researchers to study human HSCs. We recently described the spatiotemporal distribution of definitive HSCs in the early human embryo and determined that they first emerge in the aorta-gonad-mesonephros (AGM) region, specifically in the dorsal aorta (Ao) (Ivanovs et al., 2011). HSCs appear later in the yolk sac, liver, and placenta. Notably, we showed that human AGM region HSCs possess very high self-renewal potential, with each HSC producing more than 300 daughter HSCs in primary NSG recipient mice. This is reflected by the high level of human hematopoietic repopulation (reaching up to 95% of total peripheral blood leukocytes) seen in NSG mice transplanted with a single HSC from the human AGM region. To achieve the same effect with human umbilical cord blood HSCs, considerably higher numbers of HSCs need to be transplanted (Liu et al., 2010). Similar observations showing that developmentally younger fetal liver HSCs possess higher self-renewal capacity than their adult bone marrow counterparts have been made in the mouse model (Copley et al., 2013; Pawliuk et al., 1996).

During embryogenesis, various vertebrate species harbor hematopoietic cell clusters attached to the ventral wall of the Ao (Emmel, 1916; Jaffredo et al., 1998; Minot, 1912; Yokomizo and Dzierzak, 2010). Since the early HSCs and hematopoietic progenitors share common markers with endothelial cells (Taoudi et al., 2005), it is widely thought that hematopoietic cells are formed through budding from the ventral aortic endothelium and possibly reside



within the intra-aortic cell clusters (Bertrand et al., 2010; Chen et al., 2009; Kissa and Herbomel, 2010). In line with this, the expression of a number of key transcription factors and secreted molecules known to be involved in hematopoietic specification during embryogenesis, such as RUNX1, SCL, C-MYB, GATA2, GATA3, BMP4, and LMO2, is biased toward the ventral domain of the Ao (AoV) (Bertrand et al., 2005; Durand et al., 2007; Elefanty et al., 1999; Manaia et al., 2000; Marshall et al., 1999, 2000; North et al., 1999; Wilkinson et al., 2009). The sympathetic nervous system cells underlying the AoV contribute to the generation of definitive HSCs through secretion of catecholamines (Fitch et al., 2012). Furthermore, long-term repopulation studies in the mouse showed that the AoV, as opposed to the dorsal domain of the Ao (AoD), is the primary functional niche for the specification of definitive HSCs (Taoudi and Medvinsky, 2007). In vivo imaging of the zebrafish embryo has provided strong evidence that zebrafish definitive HSCs/multipotent hematopoietic progenitors emerge through the endothelio-hematopoietic transition in the AoV (Bertrand et al., 2010; Kissa and Herbomel, 2010). Although the existence of dorsoventral polarity during human hematopoietic development has already been established at the morphological and gene-expression level (Marshall et al., 1999, 2000; Minot, 1912), the ventral origin of HSCs remains a long-standing hypothesis.

Identification of the niche and phenotype of the first definitive HSCs that emerge in the human embryo is of high importance for elucidating the biology of these cells and investigating the mechanisms that underlie their development and high regenerative potential. However, this analysis is hampered by the limited availability of human embryonic tissues and the extreme rarity of HSCs in the human AGM region. Only one or two definitive HSCs can be identified at any one time in the human AGM region at the pre-liver stage of HSC development (Ivanovs et al., 2011). Assuming conservation of biological processes between species, we used knowledge obtained in the mouse model to identify the phenotype and niche of human HSCs (Medvinsky et al., 2011).

In this study, we demonstrate a strong dorsoventral polarization of hematopoietic activity in the human Ao, with the AoV containing significantly more hematopoietic cells and committed hematopoietic progenitors than the AoD. Furthermore, we show that the long-term, multilineage repopulating activity resides exclusively in the AoV, which thus represents the functional niche for developing human HSCs. We show that the CD34⁺VE-cadherin⁺CD45⁺C-KIT⁺THY-1⁺Endoglin⁺RUNX1⁺CD38^{-/lo}CD45RA⁻ phenotype defines the identity of the earliest human HSCs and provides up to 1,000-fold enrichment for HSCs in the total AGM region cell population. Our identification

of the niche and phenotype of the earliest human HSCs provides a strong foundation for analyzing the molecular mechanisms that underlie their specification and high regenerative potential.

RESULTS

Asymmetric Distribution of Hematopoietic Cells and Progenitors within the Human AGM Region

To establish the spatial distribution of hematopoietic cells within the human AGM region, we performed five independent experiments with Carnegie stage (CS) 14–17 human embryos. The expression of CD45, a panhematopoietic marker, and CD34, a marker of human adult HSCs and hematopoietic progenitors, was assessed by flow-cytometry analysis of cells isolated from the AoV, AoD, and urogenital ridges (UGRs). The AoV and the AoD included the ventral and the dorsal aortic endothelium, respectively, and the surrounding mesenchyme. The UGRs included the mesonephroi and gonadal rudiments along with the surrounding mesenchyme. All dissections (Figure S1 available online) were performed as previously described for the mouse embryo (Taoudi and Medvinsky, 2007). The AoV contained significantly more hematopoietic (CD45⁺) cells than the AoD, but a similar number compared with the UGRs (Figure 1A). We found that the AoV contained a distinct population of CD34⁺VE-cadherin⁺CD45⁺ cells, which was virtually absent in the AoD and UGRs (Figures 1B and 1D). In five independent experiments, single-cell suspensions prepared from the AoV, AoD, and UGRs were plated into methylcellulose medium supplemented with human cytokines. After 14 days, hematopoietic colonies were scored. Whereas the AoV contained 210 ± 130 colony-forming units in culture (CFU-Cs) per embryo equivalent (e.e.), the AoD and UGRs contained only 30 ± 20 and 40 ± 30 CFU-Cs per e.e., respectively (Figure 1C).

The AoV Is the Niche for the Emerging Human Definitive HSCs

We previously reported that the Ao, but not the UGRs, possess HSC activity within the human AGM region (Ivanovs et al., 2011). To determine whether the first human HSCs emerge in the AoV, we performed ten independent experiments in which the Ao of CS 15–17 human embryos was bisected into the AoV and AoD. Single-cell suspensions prepared from these tissues were individually transplanted into sublethally irradiated adult NSG recipient mice. In six out of ten experiments, we detected human high-level, long-term, multilineage hematopoietic engraftment derived exclusively from the AoV, but never from the AoD. The recipients showed progressively

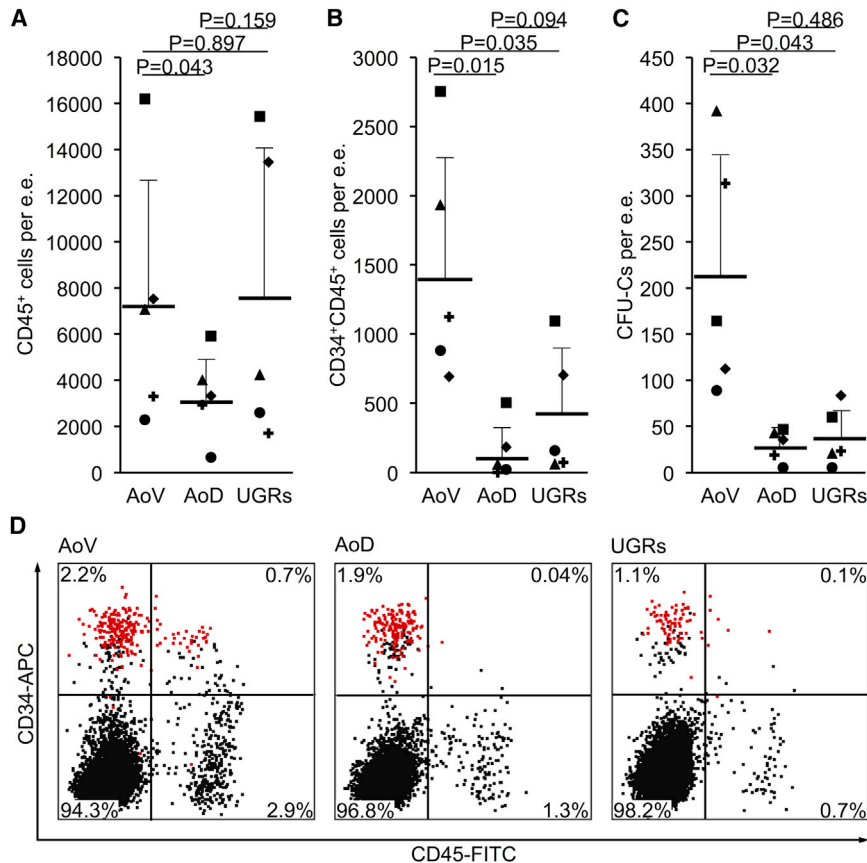


Figure 1. Dorsoventral Polarization of Hematopoietic Activity in the Human AGM Region

(A–C) AGM regions from five CS 14–17 human embryos were dissected into AoV, AoD, and UGRs (Figure S1), and single-cell suspensions were prepared from these tissues. CD45⁺ and CD34⁺CD45⁺ cell numbers per e.e. were assessed by flow cytometry. CFU-C numbers per e.e. were evaluated using methylcellulose culture. Tissues from the same embryo are represented by the same symbol on the plots. Mean values ± SD are shown.

(D) CD34, VE-cadherin, and CD45 expression in cells from the AoV, AoD, and UGRs. VE-cadherin⁺ cells are overlaid and shown in red.

increasing levels of human hematopoietic engraftment that was readily detectable in the peripheral blood, as previously described for human AGM region HSCs (Ivanovs et al., 2011).

Phenotype of Human HSCs in the AGM Region

Based on our studies in the mouse (Rybtsov et al., 2011; Taoudi et al., 2005, 2008), we were interested in testing whether human AGM region HSCs express VE-cadherin and CD45. Flow-cytometry analysis demonstrated that the human AGM region VE-cadherin⁺CD45⁺ cells are entirely positive for CD34, C-KIT, THY-1, endoglin, and RUNX1, and negative for CD38 and CD45RA (Figure 2A). This suggests the presence of HSCs in the VE-cadherin⁺CD45⁺ cell population. To confirm this, we employed magnetic cell separation using beads coupled with either anti-CD45 or anti-VE-cadherin antibodies. Antigen-positive and -negative cell populations were isolated from CS 15–17 human AGM regions and individually transplanted into sublethally irradiated adult NSG mice. In two out of three independent experiments performed with anti-human CD45 magnetic beads, HSC activity was detected exclusively within the CD45⁺ cell population (Table 1). However, in six independent experiments,

none of the recipients transplanted with the VE-cadherin⁺ or VE-cadherin⁻ cell populations were repopulated (Table 1). Although the anti-VE-cadherin antibody we used here was successfully employed to purify human fetal liver-derived HSCs (Oberlin et al., 2010), our data indicated that the VE-cadherin antibody interferes with the functional properties of HSCs isolated from the AGM region. Indeed, we found the same inhibitory effect in the mouse model (Figure S2). In three independent experiments, mouse embryonic day 11.5 (E11.5) AGM region cells were incubated with anti-mouse antibodies to CD45, VE-cadherin, or both combined, and directly transplanted into sublethally irradiated adult recipients. Unlabeled cells were transplanted as a positive control. Cells that were incubated with the anti-VE-cadherin antibody alone or in combination with the anti-CD45 antibody showed decreased hematopoietic engraftment compared with cells that were left untreated or incubated with the anti-CD45 antibody only. Therefore, VE-cadherin is a functionally important surface antigen of AGM region HSCs, and the failure of human HSCs to engraft recipient mice upon staining with anti-VE-cadherin antibody strongly suggests that they express VE-cadherin.

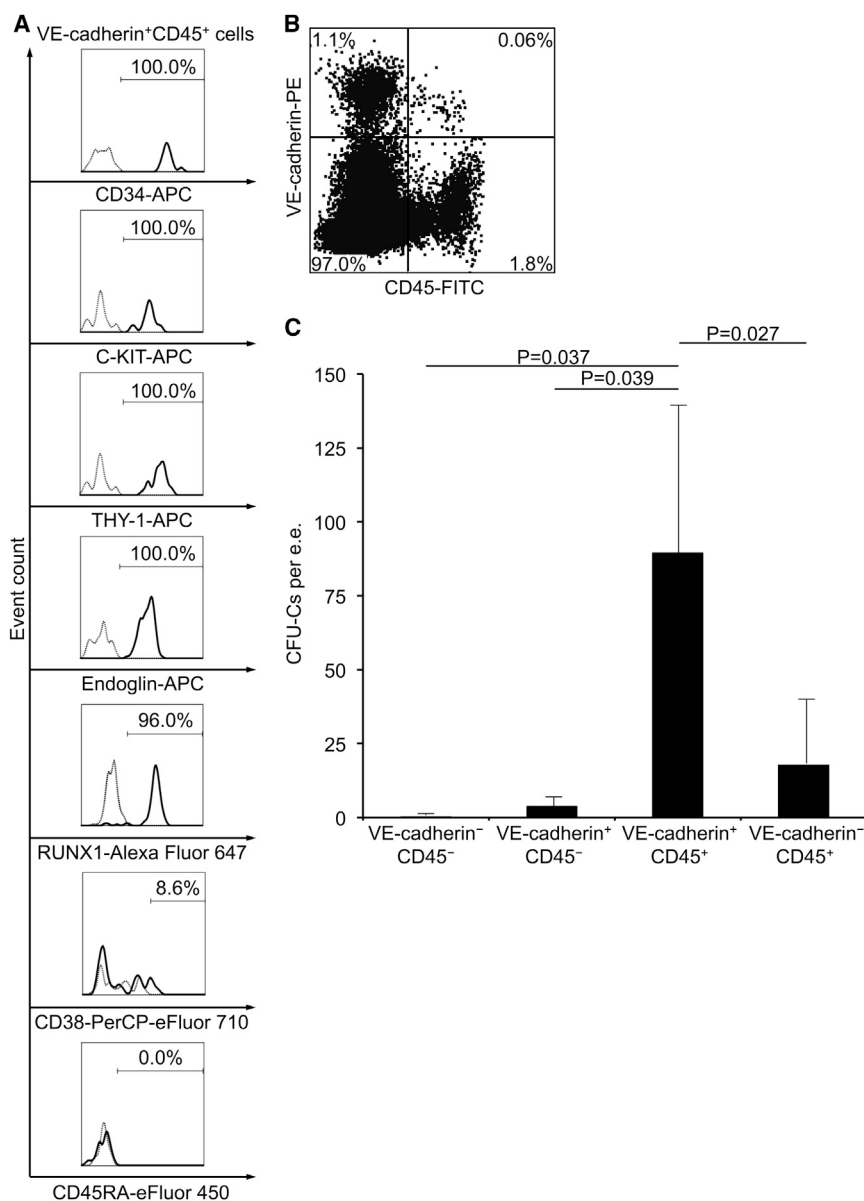


Figure 2. Phenotypic Characterization of Human AGM Region Cells

(A) CD34, C-KIT, THY-1, endoglin, RUNX1, CD38, and CD45RA expression in the VE-cadherin⁺CD45⁺ cell population. The dotted lines indicate fluorescence-minus-one controls. At least two independent experiments were performed for each antigen.

(B and C) Four cell populations were sorted from the total AGM region based on the expression of VE-cadherin and CD45 antigens, and plated in the CFU-C assay. Four independent experiments were performed. Mean values ± SD are shown. See also Table S1.

Since VE-cadherin was not suitable for purifying human AGM region-derived HSCs without affecting their ability to repopulate NSG recipient mice, we focused on CD34, which is a principal marker for human adult HSCs (Majeti et al., 2007). CD34 is also expressed in the endothelium of the developing human Ao (Oberlin et al., 2002), and therefore is a reasonable replacement for VE-cadherin. We isolated CD34⁺ and CD34⁻ cell populations using magnetic beads and individually transplanted them into sublethally irradiated adult NSG recipient mice. In two out of three experiments, HSC activity was detected exclusively within the CD34⁺ cell population (Table 1). Thus, HSCs in the human AGM region are CD34⁺CD45⁺. Our experi-

ments strongly suggest that VE-cadherin is also expressed in these cells, since incubation with the anti-VE-cadherin antibody suppresses their repopulating activity. Based on the flow-cytometry analysis data (Figure 2A), the phenotype of the first human HSCs can be extended further to CD34⁺VE-cadherin⁺CD45⁺C-KIT⁺THY-1⁺Endoglin⁺RUNX1⁺CD38^{-/lo}CD45RA⁻.

We then determined the phenotype of committed hematopoietic progenitors in the human AGM region. In four independent experiments, fluorescence-activated cell sorting (FACS) followed by the CFU-C assay showed that the vast majority of CFU-Cs reside in the VE-cadherin⁺CD45⁺ cell population. The average number of CFU-Cs

**Table 1. Phenotype of Human AGM Region HSCs**

Antigen	Cell Population Transplanted	
	Positive	Negative
CD45	2/3	0/3
VE-cadherin	0/6	0/6
CD34	2/3	0/3

Data shown are the number of AGM regions that gave human high-level, long-term, multilineage hematopoietic engraftment compared with the total number of AGM regions transplanted. See also [Figure S2](#).

in the VE-cadherin⁻CD45⁻, VE-cadherin⁺CD45⁻, VE-cadherin⁺CD45⁺, and VE-cadherin⁻CD45⁺ cell populations was 0.3 ± 0.5 , 3.8 ± 3.0 , 89.5 ± 49.9 , and 17.8 ± 21.5 CFU-Cs per e.e., respectively ([Figures 2B and 2C](#); [Table S1](#)).

DISCUSSION

In this study, we focused on the localization and phenotyping of the first HSCs that emerge in the human embryo. We found that, as in the E11.5 mouse embryo ([Taoudi and Medvinsky, 2007](#)), HSC development in the human AGM region is polarized and occurs in the AoV. However, the asymmetric distribution of hematopoietic activity in the human AGM region is more pronounced. Although CD45⁺ hematopoietic cells and CFU-Cs in the E11.5 mouse Ao are distributed more or less evenly between the AoV and AoD ([Taoudi and Medvinsky, 2007](#)), in humans these populations reside almost exclusively in the AoV.

Using the long-term repopulation assay, we demonstrated that the earliest human HSCs are CD34⁺CD45⁺. Expression of CD45 indicates full hematopoietic commitment of these newly emerged HSCs, consistent with findings in the mouse ([Taoudi et al., 2005, 2008](#)). Interestingly, CD34 is also expressed in mouse AGM region HSCs ([Sánchez et al., 1996](#)). In humans, CD34 is a principal marker for identifying adult HSCs in the bone marrow and umbilical cord blood ([Berenson et al., 1991](#); [Majeti et al., 2007](#)). CD34⁺CD45⁺ cells from the human AGM region largely coexpress VE-cadherin, an important marker for early HSCs in the mouse embryo ([Taoudi et al., 2005](#)). Our data strongly suggest that the earliest human HSCs are VE-cadherin⁺. VE-cadherin is likely to be a functionally important surface antigen of AGM region HSCs, since we demonstrated an attenuation of HSC activity following cell labeling with the anti-VE-cadherin antibody. Additional flow-cytometry analysis showed that the CD34⁺VE-cadherin⁺CD45⁺ cell population is entirely positive for major adult HSC markers such as C-KIT, THY-1, and endoglin ([Baum et al., 1992](#); [Chen et al., 2002](#); [Ikuta and Weissman,](#)

[1992](#); [Majeti et al., 2007](#); [Spangrude et al., 1988](#)), and does not express negative human adult HSC markers such as CD38 and CD45RA ([Bhatia et al., 1997](#); [Majeti et al., 2007](#)). In addition, the CD34⁺VE-cadherin⁺CD45⁺ cell population is almost entirely positive for RUNX1, which marks developing definitive HSCs in the mouse AGM region ([North et al., 2002](#)). Thus, the population of the first definitive HSCs that emerge in the human AGM region is CD34⁺VE-cadherin⁺CD45⁺C-KIT⁺THY-1⁺Endoglin⁺RUNX1⁺CD38^{-/lo}CD45RA⁻. We have found that this cell population is virtually absent from the AoD and UGRs, and contains practically all of the CFU-Cs that reside in the AGM region. Although HSCs and committed hematopoietic progenitors are highly enriched in this population, none of these markers are known to discriminate between HSCs and CFU-Cs. Similar to the case with the mouse, this remains an important unresolved issue ([Medvinsky et al., 2011](#)).

Several studies have reported the existence of rare CD34⁻ HSCs upstream of the major population of CD34⁺ HSCs in the human hematopoietic hierarchy ([Anjos-Afonso et al., 2013](#); [Bhatia et al., 1998](#)). Interestingly, the first HSCs found in the human embryo are CD34⁺ and give rise exclusively to a CD34⁺CD38^{-/lo} daughter HSC population upon xenotransplantation ([Ivanovs et al., 2011](#)). Since the subsequent downregulation of CD34 during further development cannot be excluded, precise changes in the phenotype of human HSCs require further study.

In summary, we have demonstrated that the first HSCs in the human embryo emerge in the ventral niche of the Ao and are CD34⁺VE-cadherin⁺CD45⁺C-KIT⁺THY-1⁺Endoglin⁺RUNX1⁺CD38^{-/lo}CD45RA⁻. By identifying the niche and phenotype of the first human definitive HSCs, this study lays a foundation for identifying and characterizing the molecular mechanisms that underlie the specification and high regenerative potential of the earliest human HSCs. Such knowledge may also be essential for developing protocols to derive human HSCs from embryonic and induced pluripotent stem cells for use in regenerative medicine.

EXPERIMENTAL PROCEDURES

Source and Processing of Human Embryonic Tissues

Human embryonic tissues were obtained immediately after elective medical termination of pregnancy. The study was approved by the Lothian Research Ethics Committee. Each patient gave informed consent in writing for the use of human embryonic tissues in research. The developmental stage of human embryos was determined according to the Carnegie staging system ([Ivanovs et al., 2013](#); [O'Rahilly and Muller, 1987](#)). Isolation and dissection of the AGM region was performed as described elsewhere for the mouse embryo ([Taoudi and Medvinsky, 2007](#)).



Single-cell suspensions were obtained as previously described (Ivanovs et al., 2011).

Long-Term Repopulation Assays

Experiments with mice were approved by the Animal Welfare Committee of the University of Edinburgh, Edinburgh, Scotland, UK. NSG mice were used as recipients for human HSCs. B6.SJL-*Ptprca*⁺*Pepc*^β/Boy mice (Ly5.1/Ly5.1) were used as recipients for HSCs from C57BL/6 mouse embryos (Ly5.2/Ly5.2) and bone marrow carrier cells from F1 hybrid mice (Ly5.1/Ly5.2). Cell transplantations were performed as described in the [Supplemental Experimental Procedures](#). The number of transplanted human embryonic cells was expressed in embryo equivalents (e.e.), defined as a unit of cells equivalent to the number of cells present in one tissue.

Human CFU-C Assay

CFU-C activity in human embryonic tissues was assessed by culturing in triplicate single-cell suspensions (0.1–0.05 e.e. per dish) in MethoCult H4034 Optimum Medium (StemCell Technologies) for 14 days according to the manufacturer's recommendations.

Flow-Cytometry Analysis and FACS

Surface antigen staining was performed as described in the [Supplemental Experimental Procedures](#). RUNX1 intracellular staining was performed following surface antigen staining, treatment with 0.5 μg/ml ethidium monoazide bromide, intense fluorescent light exposure, and fixation with 2% paraformaldehyde. Intracellular antibody staining and washes were performed in ice-cold Ca²⁺ and Mg²⁺-free PBS supplemented with 2% heat-inactivated fetal bovine serum and 0.4% Triton X-100 (Sigma-Aldrich).

Magnetic Cell Separation

Cell populations of interest were purified using MACS technology (Miltenyi Biotec). CD45⁺ and CD34⁺ cells were enriched using human CD45 and CD34 MicroBeads. VE-cadherin⁺ cells were isolated using anti-PE MicroBeads following cell labeling with mouse anti-human VE-cadherin-PE monoclonal antibody (clone TEA 1/31; Beckman Coulter). Magnetic cell separation was performed manually using a QuadroMACS separator and LS columns. Cell labeling, washes, and isolation were performed in accordance with the manufacturer's recommendations.

Statistical Analysis

Data are presented as mean ± SD. Comparison between groups was performed using the paired-samples t test or the Wilcoxon signed-ranks test. Statistical significance was attributed to probability value $p < 0.05$. The above statistical procedures were performed using SPSS 15.0 software (SPSS).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2014.02.004>.

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