A HUMAN BONE MARROW MESODERMAL-DERIVED CELL POPULATION WITH HEMOGENIC POTENTIAL

Saloomeh Mokhtari[1], **Evan Colletti**[2], **Weihong Yin**[1], **Chad Sanada**[3,*], **Zanetta Lamar**[1], **Paul J. Simmons**[4], **Steven Walker**[1], **Colin Bishop**[1], **Anthony Atala**[1], **Esmail D. Zanjani**[2], **Christopher D. Porada**[1], and **Graça Almeida-Porada**[1,*]

[1]Wake Forest Institute for Regenerative Medicine, Winston-Salem, NC 27157, USA

[2]Animal Biotechnology, University of Nevada Reno, Reno NV 89557, USA

[4]Institute of Molecular Medicine, University of Texas at Houston, Houston, Texas 77030, USA

Abstract

The presence, within the human bone marrow, of cells with both endothelial and hemogenic potential has been controversial. Herein, we identify, within the human fetal bone marrow, prior to establishment of hematopoiesis, a unique APLNR+, Stro-1+ cell population, coexpressing markers of early mesodermal precursors and/or hemogenic endothelium. In adult marrow, cells expressing similar markers are also found, but at very low frequency. These adult-derived cells can be extensively culture expanded *in vitro* without loss of potential, they preserve a biased hemogenic transcriptional profile, and, upon *in vitro* induction with OCT4, assume a hematopoietic phenotype. *In vivo*, these cells, upon transplantation into a fetal microenvironment, contribute to the vasculature, and generate hematopoietic cells that provide multilineage repopulation upon serial transplantation. The identification of this human somatic cell population provides novel insights into human ontogenetic hematovascular potential, which could lead to a better understanding of, and new target therapies for, malignant and nonmalignant hematologic disorders.

INTRODUCTION

During ontogeny, definitive hematopoietic stem/progenitor cells (HSC) are thought to arise from vascular endothelial cells, through an endothelial-to-hematopoietic transition, a natural process that occurs in specialized embryonic hemogenic endothelial cells.[1–3] These unique cells have been identified in tissues such as the dorsal aorta, the placenta, the yolk sac, and the cranial vitelline and umbilical arteries,[4–8] and express CD34, VEGFR2, c-Kit, and VE-Cadherin.[5, 9, 10]

*CORRESPONDING AUTHOR: Graça Almeida-Porada, M.D., Ph.D., Professor of Regenerative Medicine, Wake Forest Institute for Regenerative Medicine, 391 Technology Way, Winston-Salem, NC 27157-1083 USA., Phone: (336) 713-1630; FAX: (336) 713-7290, galmeida@wakehealth.edu.
3Currently at Fluidigm Corporation, San Francisco, CA, USA

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Studies using pluripotent stem cells demonstrated that hematovascular precursors emerge from lineage negative, APLNR[+], PDGFRα^{+} mesodermal cells following upregulation of VEGFR2 and decrease of PDGFRα expression, and that lineage commitment towards an endothelial and/or hematopoietic phenotype initiates when VE-Cadherin expression commences.[11–13]

Although the hemogenic endothelium is thought to constitute a transient population of cells within the embryo that disappears during development,[5] studies using murine endothelium have shown that, even in the adult, vascular cells are able to convert directly to immunocompetent HSC after forced expression of *Fosb*, *Gfi1*, *Runx1*, and *Spi1* transcription factors,[14] and that other cells present within the murine bone marrow, such as the very small embryonic-like stem cells, differentiate into the hematopoietic lineage after coculture over OP9 stromal cells.[15]

In humans, it has been reported that extremely rare subsets of both fetal[16] and adult[17] bone marrow endothelial cells are endowed with hematopoietic differentiation capacity. Thus, it is possible that the emerging bone marrow vascular network, which provides a suitable environment for the lodging of HSC emigrating from other fetal sites, contains cells with a hemato-endothelial signature that persist as a vestigial population of cells.

Here, we delineate the phenotype of cells during bone marrow ontogeny, and show that a subpopulation of cells express the G-protein–coupled *APELIN* receptor (APLNR), a marker of early mesoderm enriched for hemangioblast colony-forming cells, and a regulator of early human hematopoiesis.[18] In addition, we demonstrate that the vast majority of APLNR+ cells co-express Stro-1+,[19, 20] a marker of endothelial[21] and perivascular cells,[22–24] and that the APLNR+ Stro-1+ subpopulation expresses VEGFR2 and VE-cadherin, and low levels of PDGFRα. Moreover, we determined that Stro-1+, VE-cadherin+ cells are present within the developing marrow vasculature, and that, although their frequency decreases as gestation progresses, in adulthood they persist at constant levels as a rare population of cells within the bone marrow. We additionally established that cultured/expanded adult Stro1+ isolated primary cells (SIPs), although unable to generate hematopoietic colonies in semisolid media, assume a hematopoietic phenotype when induced with OCT4. In addition, SIPs, when transplanted into a fetal environment, lodge within the vasculature and perivascular compartment, and after up-regulating endogenous OCT4, they generate hematopoietic cells that are capable of serial hematopoietic reconstitution, with generation of both myeloid and lymphoid cells. Transplantation of clonally-derived SIPs into fetal animals also confirmed these results. These data have shed light on the development of vascular niches during bone marrow development, and could thus lead to a better understanding of normal hematopoiesis and/or onset of malignant and nonmalignant hematologic disorders.

MATERIALS AND METHODS

Analysis of human fetal and adult bone marrow

Human fetal bone tissues were obtained from Advanced Bioscience Resources (Alameda, CA). Human adult bone marrow (BM) was obtained from healthy adult donors after informed consent, or from commercial sources (AllCells, LLC, Alameda, CA). Fetal and

adult tissues were obtained according to guidelines from the Office of Human Research Protection at Wake Forest Health Sciences, and University of Nevada, Reno.

Flow cytometric analysis of adult or fetal BM was performed using methods described in Supplementary Materials and Methods (SMM). Isolation and culture of BM-derived Stro1+ isolated primary cells (SIPs) and clonal cell populations is also detailed in SMM. In short, SIPs were isolated based on Stro-1 positivity, CD45 and GlyA negativity, and fibronectin adhesion, and clonally-derived SIPs were sorted into fibronectin-coated 96-well plates by single cell deposition using a FACSVantage (BD Biosciences, San Jose, CA). 24 hours later, wells were visually inspected by phase contrast microscopy (Olympus IX70, Melville, NY) to confirm the presence of a single cell/well. Only wells containing single cells were used to establish the clones employed in these studies. The number of wells positive for cellular growth that initially contained a single cell was 83%.

Confocal Microscopy

A detailed description of methods used appears in SMM. Confocal fluorescence images were acquired with an Olympus Fluoview 1000 or FV1200 SPECTRAL Laser scanning confocal microscope with 488, 594, and 647 nm laser excitations and an Olympus UPlanFLN- 40×/1.30 oil objective. Confocal images were taken as z-stacks with 5–9 slices per image before being projected as 2D images. Image acquisition and primary image processing were done using Fluo View™ software.

Tube formation assay

Tube formation assays were performed in 24-well plates by seeding 1×10^4 cells/well onto 300μl of polymerized Matrigel (BD Biosciences, Bedford, MA) at 37°C, in EGM-2 supplemented with an additional 10% FBS, as described in SMM.

Transplantation of SIPs into fetal sheep recipients

Fetal sheep (n=41) were injected intraperitoneally by ultrasound-guided transabdominal percutaneous injection,[28, 29] with SIPs populations in QBSF serum-free media (Quality Biological, Gaithersburg, MD), at the doses indicated in the results section, at 55–62 days of gestation, and according to University of Nevada approved Institutional Animal Care and Use Committee (IACUC) guidelines.

Assessment of human donor hematopoietic cell engraftment

BM and PB from animals transplanted with human cells were analyzed for the presence of donor (human) hematopoietic cells by flow cytometry using monoclonal antibodies and methods described in SMM.

Assessment of human SIPs engraftment in secondary recipients

Stro-1+ human cells were isolated from bone marrow of secondary recipients using magnetic sorting, as described in SMM. Sorted cells were used to extract DNA to perform PCR, or for immunostaining. After confirmation by PCR that samples were positive for β-actin, PCR was performed using human-specific HLA-DQα[30] and/or GAPDH primers[31] as detailed in SMM.

Transplantation of CD34+CD45+ cells isolated from bone marrow of primary sheep SIPs recipients into NOD/scid/γ(c)(–/–) (NSG) mice, and flow cytometric analysis of transplanted NSG Mice

Newborn NSG mice (1–3 day old) were irradiated with 100 cGy and injected intrahepatically, as previously described,[32] with human *CD34+CD45+* cells isolated from bone marrow of primary sheep SIPs recipients. Details of the transplant and analysis can be found in SMM.

Adult SIPs cell transduction and induction of cellular reprogramming

Plasmid construction, lentiviral vector generation for cellular reprograming, and culture details[26] can be found in detail in SMM.

Statistics and data analysis

Experimental results are presented as the mean plus/minus the standard error of the mean (SEM). We used GraphPad Prism 6 (GraphPad Software) for statistical analysis. Comparisons between experimental results were determined by two-sided non-paired Student's *t*-test analysis. A *p* value <0.05 was considered statistically significant. We also validated the power of our statistical analysis by using the two-sided Wilcoxon Rank Sum Test for independent samples.

RESULTS

During human development, the APLNR+, Stro-1+ population contains cells with a phenotype consistent with that of hemogenic endothelial precursors

During the commitment of human pluripotent stem cells to the hematopoietic lineage, apelin receptor (APLNR), vascular endothelial growth factor receptor 2 (VEGFR2)[11, 18], and platelet-derived growth factor receptor-α (PDGFRα)[33] have been used to define the early mesodermal precursors with the potential to give rise to endothelial, mesenchymal, and hematopoietic cells. Subsequent mesodermal commitment to the endothelial and hematopoietic lineages proceeds through VE-cadherin+ intermediates.[34] Within the human bone marrow, stromal elements with the capacity to transfer the hematopoietic microenvironment *in vitro* were reported to express Stro-1, an antigen discovered with an antibody generated by immunizing mice with human 34+ cells.[19]

Confocal microscopy of fetal bone marrow at 10±1.5 gestational weeks (gw) demonstrated that APLNR+ cells are present in the inner part of the developing fetal marrow and express Stro-1 (Fig. 1a). The continued presence of APLNR+, Stro-1+ cells was confirmed by flow cytometric analysis of cells freshly isolated from 18±1.5gw (n=3) donors (Fig. 1b). More than 90% of APLNR+, Stro-1+ cells were VE-Cadherin+, most co-expressed VEGFR2, and, in addition, a smaller percentage was PDGFRα positive (Fig. 1b). By contrast, less than half of the APLNR-, Stro-1+ cells expressed VE-Cadherin, only a small fraction expressed VEGFR2, and none were positive for PDGFRα after subtracting non-specific staining seen with the isotype control (Fig. 1b). Therefore, during fetal development, the APLNR+, Stro-1+ population contains cells with a phenotype consistent with that of hemogenic endothelial precursors.

In addition to VEGFR2 and VE-Cadherin,[13, 35] CD34[36] expression has also been used to identify populations of cells with a hemato-endothelial phenotype. Confocal analysis demonstrated that CD34+,VEGFR2+,Stro-1+ cells localize within vascular structures of fetal bone (n=3) at 10±1.5gw (Fig. 2a). Later in gestation, at 18±1.5gw (n=3) (Fig. 2b), cells co-expressing Stro-1, CD34, and VEGFR2 were also identified in specific locations within the vascular niche. Flow cytometric evaluation of freshly isolated Stro-1+ cells at 18±1.5gw (n=3) confirmed expression of VEGFR2 in 7.6–39.6% of CD34+,Stro-1+ cells (Fig. 2c). Quantification of Stro-1+ and VE-Cadherin+ cells at different gw showed that, throughout gestation, VE-Cadherin expression mirrored that of Stro-1, with both being highly expressed early in gestation, and decreasing with fetal bone maturation (Fig. 3 a,b) (n=4). The percentage of Stro-1+ cells at 10±1.5gw was 27.47±0.49% and then decreased progressively over time, to reach 7.81±0.6% at 20gw (Fig. 3b), as the marrow matured to become fully hematopoietic. Confirmation of the predominantly vascular nature of the 10±1.5gw marrow, and its evolution to a hematopoietic organ by 18±1.5gw, was also obtained by flow cytometric analysis of freshly isolated, unfractionated fetal bone marrow, demonstrating that it was only at this later time point that the majority of CD34+ cells were also CD45+ (Supplementary Fig. 1a).

We further investigated whether Stro-1+ subpopulations of cells also expressed markers of established endothelium. Confocal microscopy of fetal bone at 10±1.5gw confirmed the presence of CD31+Stro-1+ cells lining vascular areas (Supplementary Fig. 1b). Evaluation of freshly isolated Stro-1+ cells from later stages in gestation (18±1.5gw; n=3), showed that 23.9–42.3% of Stro-1+,CD34+ cells co-express CD31, that 4–19.7% of Stro-1+,CD31+,CD34- cells are present, and that 7.8–19.9% of Stro-1+,CD34+ cells express the endothelial/perivascular marker CD146 (Supplementary Fig. 1c).

In order to further characterize Stro-1+ cells during development, we investigated whether these cells expressed markers associated with other stem cell phenotypes.

As can be seen in Supplementary Fig. 1c, Stro-1+, CD34+ cells express CD49f (30.8–38.3%), a marker of primitive HSC,[37] as well as CD90 and CD117, markers of primitive stem cell populations within the hematopoietic and vascular lineages.

Within the freshly isolated human adult bone marrow, APLNR+, Stro-1+, VE-Cadherin+ cells constitute a rarer population of cells

We next evaluated whether human adult bone marrow APLNR+ cells expressed markers identical to those found in fetal APLNR+ cells during development. Flow cytometric analysis of freshly isolated BMMNC confirmed that APLNR+ Stro-1+ cells are still present (n=3), but are rarer in the adult than during development (Fig. 3c). Furthermore, in contrast to their fetal counterpart, only APLNR+ Stro-1+cells expressed VE-Cadherin, and VEGFR2 expression in adult APLNR+ Stro-1+ cells was significantly decreased. Also, evaluation of adult Stro-1+ cells revealed loss of expression of markers associated with primitive stem cell populations and the vascular lineage. Specifically, when compared with fetal cells (Supplementary Fig 1c) fewer Stro-1+ cells expressed CD117 and CD31, most cells that expressed CD34 were also CD31 positive, and only a very small percentage of Stro-1+ cells expressed CD49f (n=3) (Supplementary Fig. 2).

Phenotypic and functional comparison of cultured fetal and adult S̲tro1+ I̲solated P̲rimary cell̲s (SIPs)

Both fetal and adult Stro-1+ cells are rare in freshly isolated bone marrow, but they can be isolated and expanded (SIPs), as adherent populations to significant numbers. Therefore, we next determined the phenotype and function of these cells after *in vitro* culture. Fetal and adult SIPs displayed similar phenotypic markers (Fig. 4a, Supplementary Fig 3a, and Supplementary Results), but CD34 mRNA transcripts were only detectable in fetal SIPs (Fig. 4b). TEM images comparing the morphology of these cells can be seen in Supplementary Fig. 3d. Tube formation assays demonstrated that adult and fetal SIPs at the same passage (P4), formed capillary tubes, but fetal cells were significantly more efficient than their adult counterparts (Fig. 4c). In addition, both cultured fetal and adult Stro-1+ cells expressed UEA-1, EphB2, and EphrinB2, markers associated with endothelial cells and angiogenesis[38] (Supplementary Fig. 3c).

Cultured Adult SIPs generate hematopoietic cells in vivo

Transcriptome analysis of adult SIPs demonstrated that they express some hemangioblast- and hematopoietic-associated genes (Supplementary Results and Supplementary Fig. 3d.) Therefore, we next investigated the ability of SIPs to generate hematopoietic cells *in vivo.* SIPs expanded *in vitro* (P3–5) were transplanted into a total of 10 sheep fetuses at the following doses: 3.25×10^5/fetus (n=3), 1.3×10^6/fetus (n=3), and 1×10^7/fetus (n=4). Analysis of hematopoietic engraftment, as determined by the total percentage of human myeloid and lymphoid cells at 2 months post-transplant, is shown in Fig. 5a. In the PB, the percentage of human hematopoietic cells varied from 0.47 to 6.54%, and in BM from 0.68 to 10.72%. While all transplanted animals harbored donor-derived lymphocytes, human myeloid and lymphoid cells were concurrently detected in 9 out of 10 of the recipients. Lymphoid and myeloid engraftment, with reconstitution of both granulocytic and erythroid compartments, was detected in 50% of the animals, and 90% of recipients had donor-derived lymphoid and erythroid cells. Human CD34+ cells were found in 60% of the transplanted animals, at levels ranging from 0.02 to 1.65%, and their levels did not directly correlate with either overall or multilineage engraftment. The overall percentage of engraftment within the different hematopoietic lineages of these primary recipients is detailed in the left bar of Fig. 5b, and representative images of flow cytometric analysis appear in Supplementary Figs. 4 and 5a. RFP-positive cells could readily be detected in the PB of in one additional animal that received SIPs transduced with an MSCV-Neo-RFP vector. (Supplementary Fig. 5b).

In order to investigate whether serially-transplantable HSC were being generated from SIPs within primary recipients, we performed serial transplantation studies, in which BM from primary SIPs recipients was transplanted into 8 secondary fetal sheep recipients.

Analysis of human hematopoietic engraftment in PB and BM in secondary recipients, as determined by the percentage of human myeloid and lymphoid cells at 2 months post-transplant, is shown in Fig. 5c. Human hematopoietic cells were detected in the PB and BM of all of the transplanted animals. The range of human hematopoietic cells was 2.17–6.86% and 0.46–3.14% in the PB and BM, respectively. All transplanted animals harbored donor-derived lymphocytes and erythroid cells, while 80% had lymphocytes, granulocytes, and

erythrocytes. Human CD34+ cells (0.09–1.07%) were present in 60% of the transplanted animals. The overall percentage of engraftment within the different hematopoietic lineages of the secondary recipients is detailed in the right bar of Fig. 5b and in Supplementary Fig. 4.

In addition, human CD34+CD45+ cells, isolated from frozen bone marrow of primary SIPs recipients, were capable of engrafting 2 of 3 NSG mice for at least 8 wks, as assessed by the presence of human CD45+ cells (Fig. 6a and Supplementary Results)

SIPs persist in the bone marrow of the transplanted animals

To determine if all SIPs converted to hematopoietic cells, or some had persisted in the bone marrow of the transplanted animals as SIPs, we collected BM from 2 primary recipients that had received SIPs transduced with a lentivector encoding GFP, and transplanted these cells into 2 additional secondary recipients at a dose of 3.7×10^6 cells/fetus. Stro-1+ cells were sorted from the BM of secondary recipients (n=2) at 30 months post-transplant, and grown in culture. As can be seen in Fig. 6b, GFP-positive human SIPs were detected in culture. The human origin of these cells was further confirmed by PCR analysis, using human-specific primers for GAPDH.

Evaluation of clonally-derived SIPs for their ability to generate vascular cells and HSC after transplantation into fetal sheep

To show that the observed donor-derived hematopoiesis did not arise from undetectable levels of hematopoietic cells in culture, clonally-derived SIPs, obtained by deposition of single cells in 96 well plates, were expanded until sufficient cells were obtained. Phenotypic characterization of the various resultant clones demonstrated these cells to be similar to non-clonally derived SIPs (data not shown). Each clonally-derived SIP population was transplanted into one fetal sheep at a dose of 10^6cells/fetus (n=8). Evaluation of the recipients at 75 days post-transplantation for the presence of human hematopoiesis showed that all 8 transplanted SIP clones generated hematopoietic cells (Fig. 7a, and Supplementary Fig. 4) in the BM (1.25–7.63%), and PB (8–13.73%), including both myeloid and lymphoid cells, as determined by flow cytometry (Fig. 7b left column). In 88% of the animals, donor-derived contribution to the granulocytic and erythroid compartments was also detected, and CD34$^+$ cells were detected in all transplanted animals (0.22±0.05%), (Supplementary Fig. 5a lower panel). PCR analysis, using human-specific primers for HLA-DQα, demonstrated that methylcellulose colonies (CFC) grown from primary recipients that received clonally-derived SIPs were of human origin (Supplementary Fig. 5c).

Transplantation into secondary fetal sheep recipients (n=12) confirmed that the hematopoietic cells generated from clonally-derived SIPs in the primary fetal sheep recipients were able to engraft secondary recipients; the total percentage of human cells in the PB (0.57–35%) and BM (0.45–25%), as detected by flow cytometry, is depicted in Fig. 7c and Supplementary Fig. 4. CD34+ cells were present in 60% of these secondary recipients (0.53±0.14), and the animals with the highest levels of engraftment also exhibited expansion of either the erythroid (Gly-A) or lymphoid (CD7) lineage (Fig. 7b right column).

We also investigated whether clonally-derived SIPs could contribute to both the vascular niche and hematopoietic cells after transplant. Confocal microscopy on sections of bone marrow harvested from animals transplanted with a clone of SIPs that had been transduced with a lentiviral vector encoding GFP demonstrate that Stro-1+GFP+ cells can readily be identified contributing to the vasculature of the transplanted animal (Supplementary Fig. 6a). Flow cytometric analysis using an antibody against GFP demonstrated GFP-positive cells in the BM of these animals (Supplementary Fig. 6b). Confirmation this clonal cell population also gave rise to cells within the hematopoietic lineage was obtained by flow cytometric analysis, which demonstrated the presence of human hematopoietic cells (Supplementary Fig. 6c).

Oct4 induces SIPs towards a hematopoietic phenotype in vitro

After determining that human OCT4 was expressed in the marrow of all transplanted animals, to varying degrees (Supplementary Figure 7a), we next performed *in vitro* studies to examine whether over-expression of OCT4 could induce SIPs towards a hematopoietic phenotype.[26] Data presented in the supplementary results, and Supplementary Figure 7b,c demonstrate that *in vitro* induction of SIPs using a lentivector encoding OCT4 led the generation of colonies co-expressing CD45 and CD34.

DISCUSSION

In humans, little evidence has been provided that a cell population with hemogenic properties exists within the fetal bone marrow, but this possibility has not been entirely excluded.[39] Morphologic studies investigating human fetal bone marrow hematopoietic ontogeny have shown that the development of the vascular bed occurs between 9–10.5gw, and precedes establishment of hematopoiesis.[40, 41] Here, we provide the first evidence that, at these early time points of gestation, APLNR, a marker of mesangiohematopoietic mesoderm[11, 18] is expressed by cells that also express Stro-1 in the inner part of the developing bone marrow. APLNR+ Stro-1+ cells are also VEGFR2^{bright},PDGFRα$^{Low/-}$, a repertoire of markers associated with hematovascular mesodermal precursors. The concomitant expression of VE-Cadherin, a marker connected with the emergence of hemogenic endothelial precursors[13] by APLNR+, Stro-1+ cells, indicates that these cells have a phenotype fitting that of late hematovascular mesodermal precursors/emerging hemogenic endothelial precursors.

The use of CD34 as another marker of hematovascular cells,[36] to delineate *in vivo*, the sites where Stro-1+ cells localize during fetal BM development, demonstrated that Stro-1+,VEGFR2+,CD34+ are present within the vascular niche of the developing bone. We also showed, for the first time, that although the percentage of Stro-1+ cells decreases during development to constitute a small population within the adult bone marrow, a subpopulation of Stro-1+ cells within the adult still expresses APLNR, VE-Cadherin, and VEGFR2.

After *ex-vivo* expansion, characterization of fetal and adult SIPs showed that expression of VEGFR2+ and VE-Cadherin was lost, when compared to freshly isolated cells, while the expression of PDGFRα was increased. Nevertheless, neither fetal nor adult SIPs were able to produce hematopoietic colonies upon methylcellulose culture, which is in agreement with

what has previously been described for hematovascular mesodermal precursors and hemogenic endothelial precursors.[13] Prior studies showed that the hematopoietic and endothelial potential of hematovascular mesodermal precursors could be demonstrated after co-culture with OP9 cells.[13, 15] Because co-culture of SIPs with OP9 cells did not reveal their hematopoietic potential, here we used an *in vivo fetal* model to test the hemogenic and vasculogenic potential of *in vitro* expanded SIPs. These studies show that these adult cells generated hematopoietic cells *in vivo*, and that SIPs were capable of multilineage hematopoietic reconstitution of primary and secondary sheep and mice recipients. Further support that the inherent hemogenic potential is unique to SIPs comes from studies in which we showed that transplantation of other human BM-derived stromal cells which were not isolated based upon Stro-1-positivity, were unable to generate detectable levels of hematopoietic cells in this same fetal model.[42] In addition, *in vitro* induction of SIPs using a lentivector encoding OCT4 led to the generation of colonies co-expressing CD45 and CD34. The fact that, in SIPs-transplanted recipients, there was a strong bias towards lymphoid engraftment, despite the presence of myeloid cells, raised the possibility of the contamination of SIPs with primitive long-lasting lymphoid cells. The data demonstrating that clonally-derived SIPs were also able to generate HSC that were serially-transplantable rules out this possibility, and provides further proof that SIPs have the ability to convert to hematopoietic cells, if primed with the necessary factors. In addition, we show for the first time, that clonally-derived SIPs also contribute to the vascular elements.

In conclusion, these studies have demonstrated the existence of an expandable population of adult human somatic cells, whose ontogenetic phenotype parallels that described for hematovascular mesodermal precursors and/or hemogenic endothelial precursors,[13] and show these cells have the ability to contribute to vascularization and generate hematopoietic cells upon transplantation. The identification of this human somatic cell population provides novel insights into human ontogenetic hematovascular potential, which could lead to a better understanding of malignant and nonmalignant hematologic disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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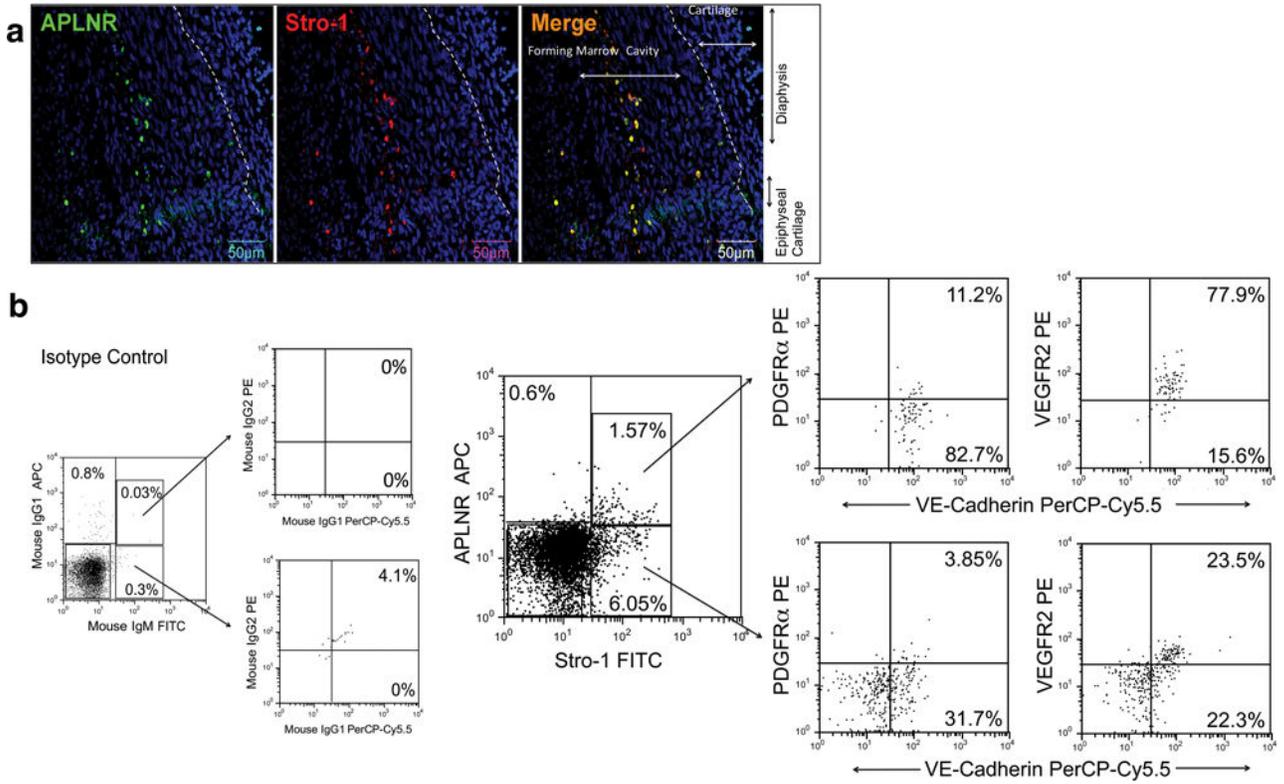
Figure 1. APLNR+, Stro-1+ population contains cells with a phenotype consistent with that of hematovascular mesodermal precursors and/or hemogenic endothelial precursors
(**a**) APLNR+ Stro-1+ cells can be identified at 10±1.5gw in the inner part of the developing bone marrow (n=3). DAPI (blue) labels all nuclei. (**b**) Representative dot plots of flow cytometric evaluation of freshly isolated fetal BMMNC, demonstrating that APLNR+ cells are present, and that the majority of these cells express Stro-1 (n=3). Stro-1+ APLNR+ cells are VE-Cadherin+, express VEGFR2, and a small percentage also express PDGFRα. By contrast, Stro-1+ APLNR- cells contain a mixture of VE-Cadherin positive and negative cells, and only a small fraction express VEGFR2. Percentages depicted in the images are before subtraction of respective isotype controls (n=3). Percentage of background fluorescence obtained when staining the same population of cells with appropriate isotype control is shown in the leftmost panel.
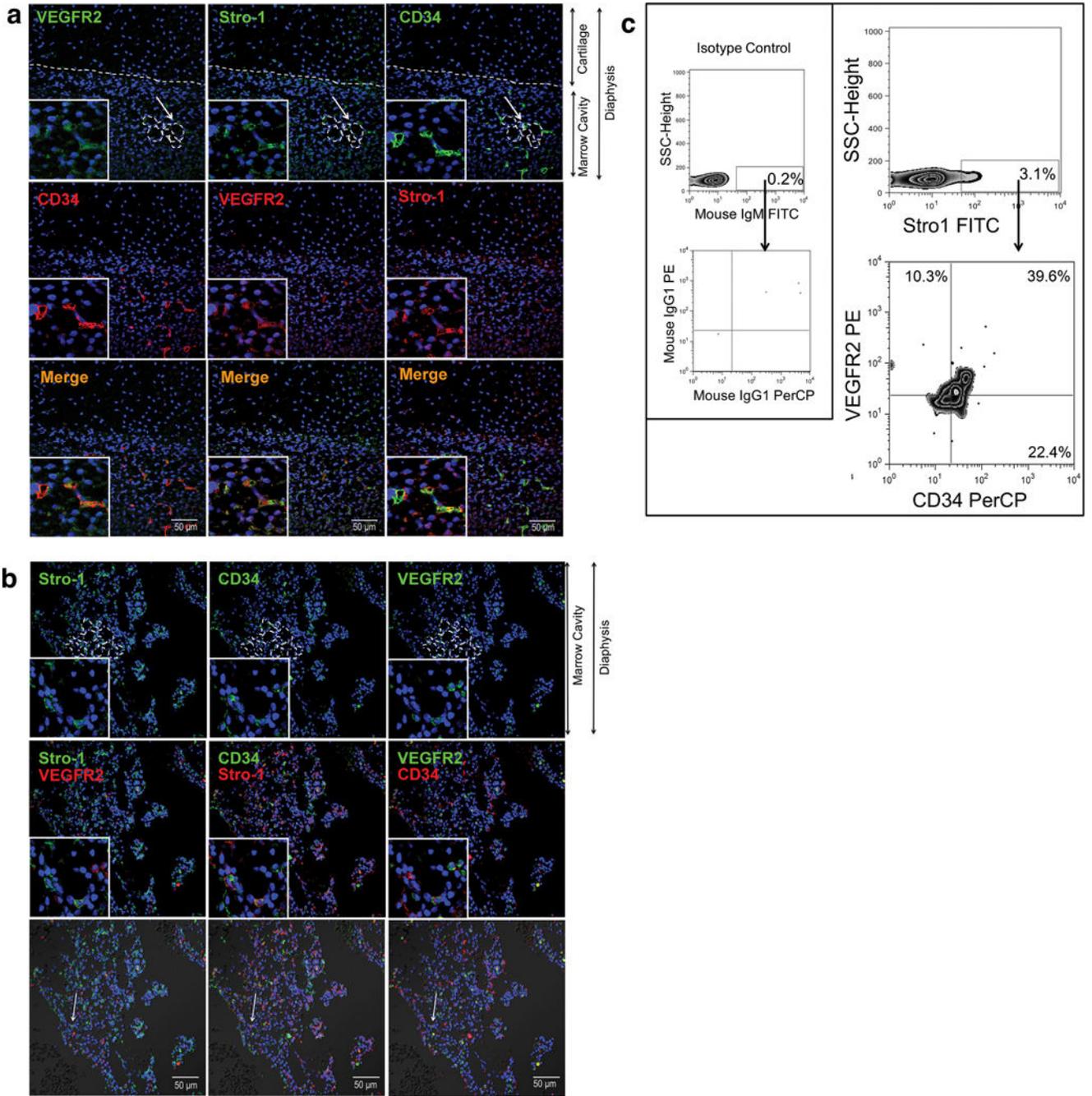
Figure 2. CD34+,VEGFR2+,Stro-1+ cells localize within vascular structures of the fetal bone
(**a, b**) Staining was performed using 3 different antibodies labeled with respective Alexa Fluor Dyes: Stro-1 (Alexa Fluor 647), VEGFR2 (Alexa Fluor 488), and CD34 (Alexa Fluor 594). This allowed us to visualize Stro-1, VEGFR2, and CD34 individually in rows 1 and 2, and, in combination in row 3 to determine co-expression (merged images) in vascular structures (represented as dotted lines), at (a) 10±1.5gw (n=3) and at (b)18±1.5gw (n=3). DAPI (blue) labels all nuclei. Arrows indicate features enlarged in insets. (**c**) Representative flow cytometric analysis (18±1.5gw) confirming that Stro-1+ cells co-express CD34 and

VEGFR2 (n=3; 18±1.5gw). Percentages depicted in zebra plots were calculated after subtraction of respective isotype control. Isotype control is shown in left panel.
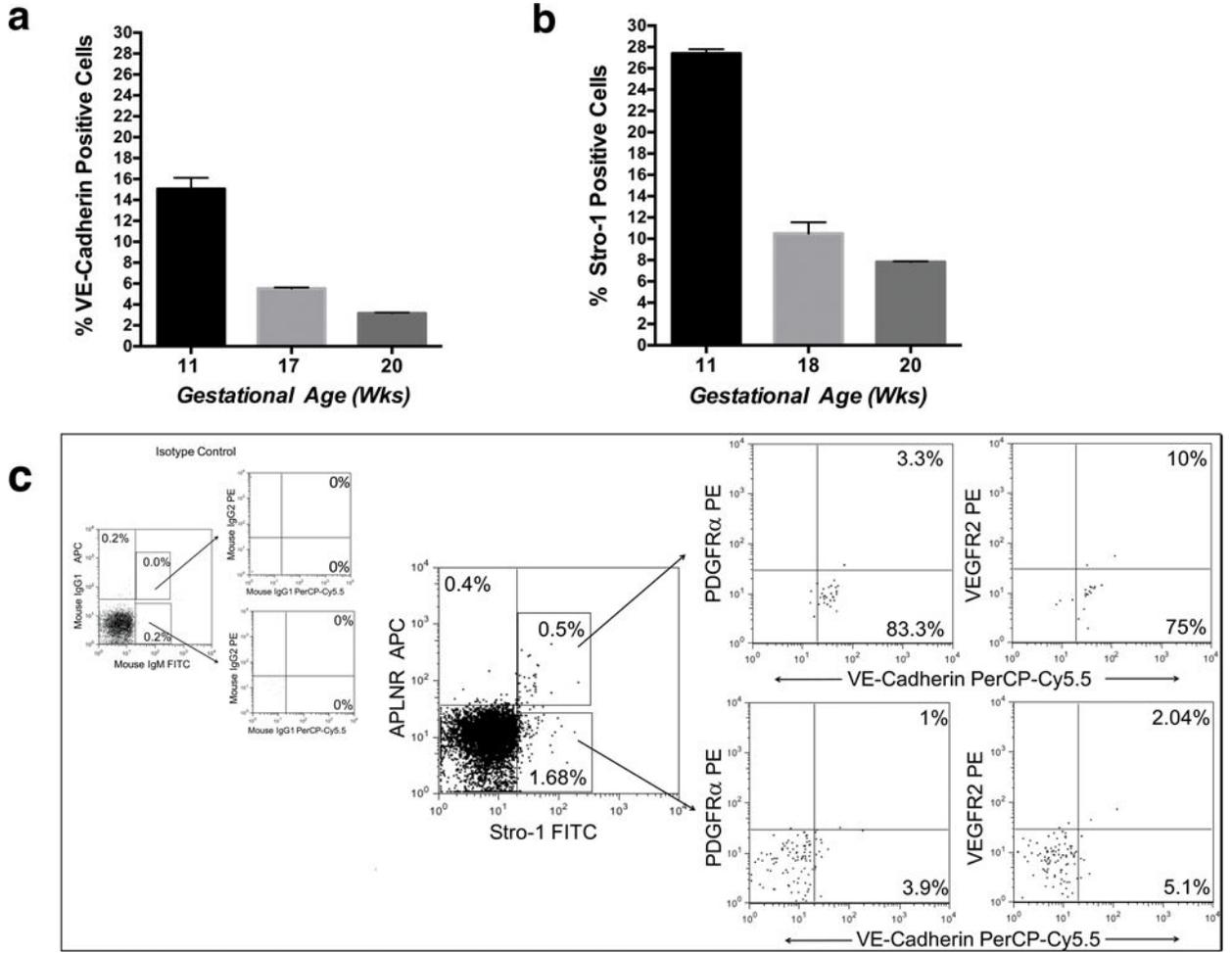
Figure 3. Expression of VE-Cadherin during development. In the freshly isolated adult bone marrow, APLNR+, Stro-1+, VE-Cadherin+ cells constitute a rarer population of cells
(**a**) Percentage of VE-Cadherin+ (n=3), and (**b**) Stro-1+ cells (n=3), during marrow ontogeny. (Error bars reflect SEM). (**c**) Representative dot plots of flow cytometric evaluation of freshly isolated adult BMMNC demonstrating that Stro1+ APLNR+ cells are still present (n=3); appropriate isotype controls are shown on the left. Stro-1+APLNR+ cells expressed VE-Cadherin, and few cells expressed VEGFR2. Percentages depicted in the images are shown after subtraction of respective isotype controls.
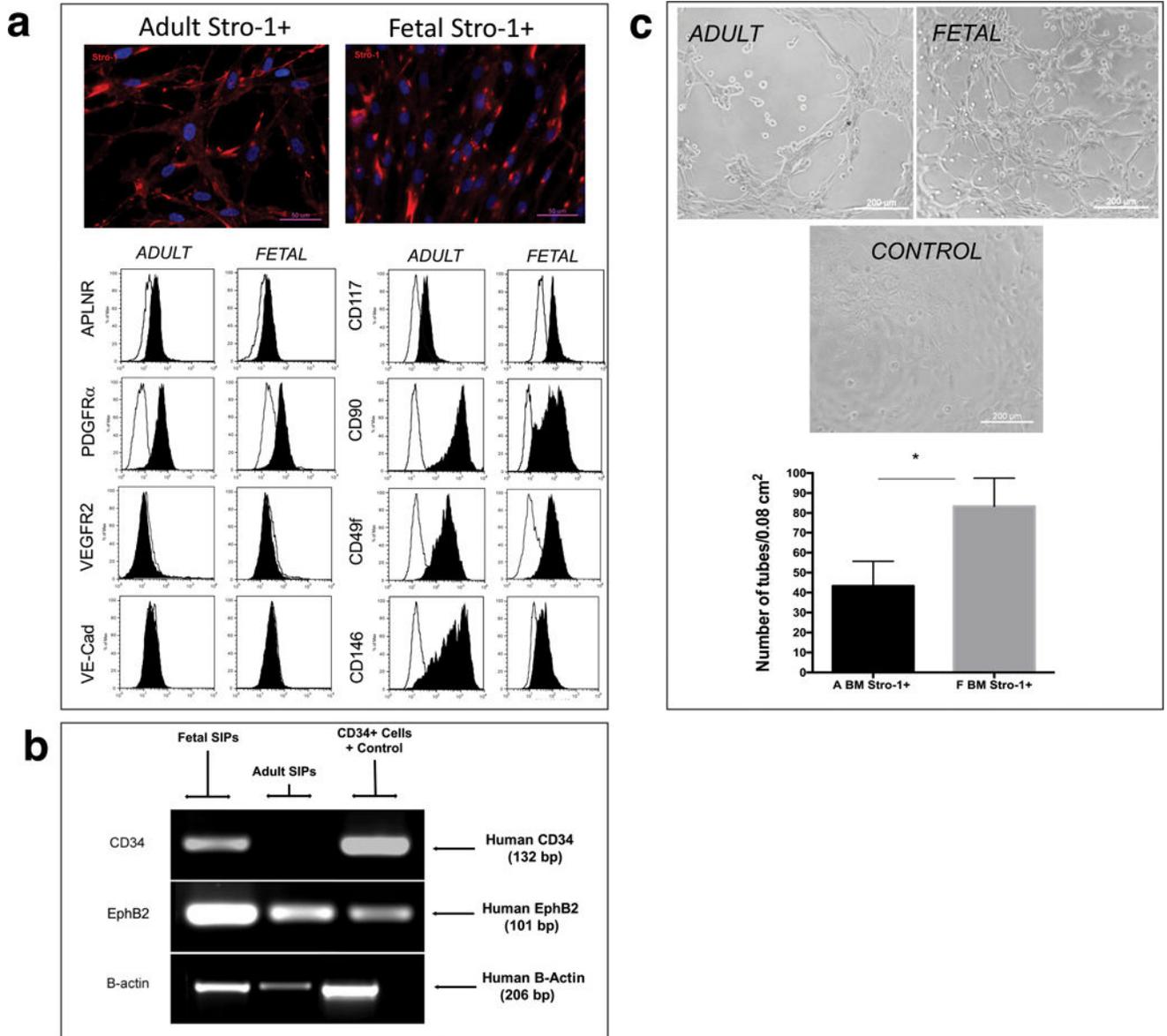
Figure 4. Comparison of cultured fetal and adult SIPs

(**a**) Immunofluorescence and Flow cytometric analysis of Stro-1+ cultured fetal and adult cells at P4 are depicted (n=3). The black filled histogram shows fluorescent data for each specific marker, and the unfilled line marks respective isotype controls. Cell populations were considered positive if the ratio between the Median Fluorescence Intensity (MFI) for the specific marker and the MFI of the isotype control ≥ 1.5. (**b**) RT-PCR analysis, using human-specific primers for CD34, demonstrated that fetal, but not adult, SIPs expressed CD34. Amplification of the same samples with primers specific for human EphB2 and B-actin confirmed the presence amplifiable RNA. For each set of primers samples were run simultaneously in the same gel, but gel was cropped to improve clarity, and to show amplification of samples specific to fetal and adult BM-derived SIPs. (**c**) Tube formation assays confirmed that, at the same passage (P4), both cell populations were able to form

capillary tubes. Cells initiated tubule formation at 2–4h of culture, which fully developed by 6h, with the presence of nodes of   4 branches; images were acquired on a Ziess Axiovert 200M and 10X magnification. Quantification of number of branch sites/nodes demonstrated fetal cells were significantly more efficient at generating tubes than their adult counterparts (n=3).
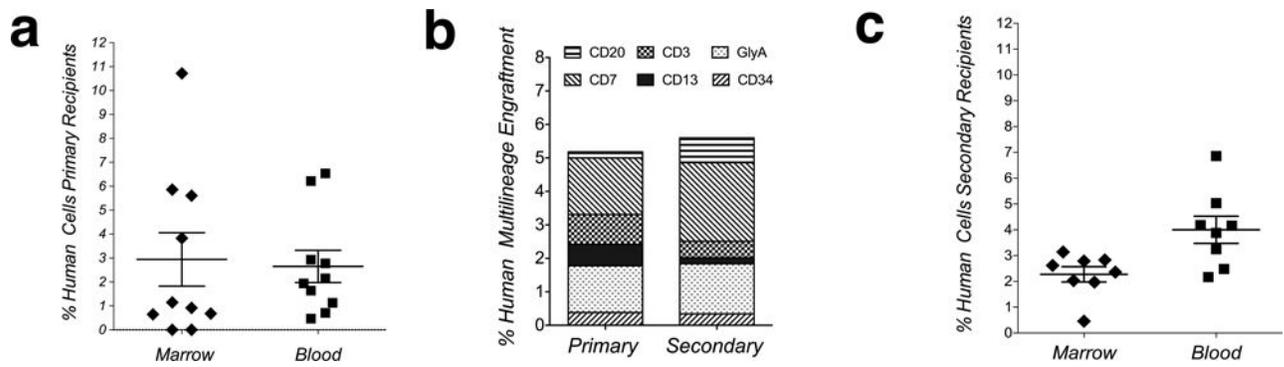
Figure 5. Adult SIPs are serially transplantable, and converted in vivo to HSC with multilineage differentiation capability in primary and secondary recipients

(a) Percentage of total human hematopoietic (myeloid and lymphoid) engraftment at 2 months post-transplant in animals transplanted with SIPs (n=10); each mark represents levels of engraftment within one animal. **(b)** Overall percentage of engraftment within the different hematopoietic lineages. **(c)** HSC generated in primary recipients are serially transplantable. Analysis of human hematopoietic engraftment in blood and marrow in secondary recipients (n=8), as determined by the percentage of human myeloid and lymphoid cells at 2 months post-transplant, is shown.
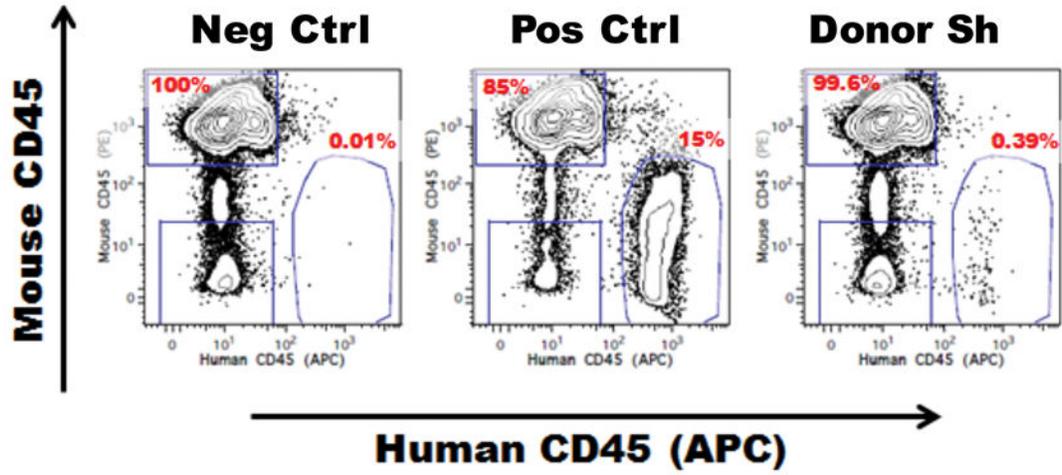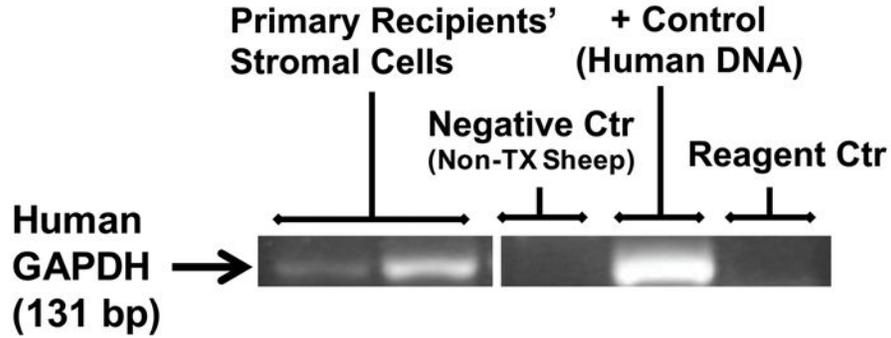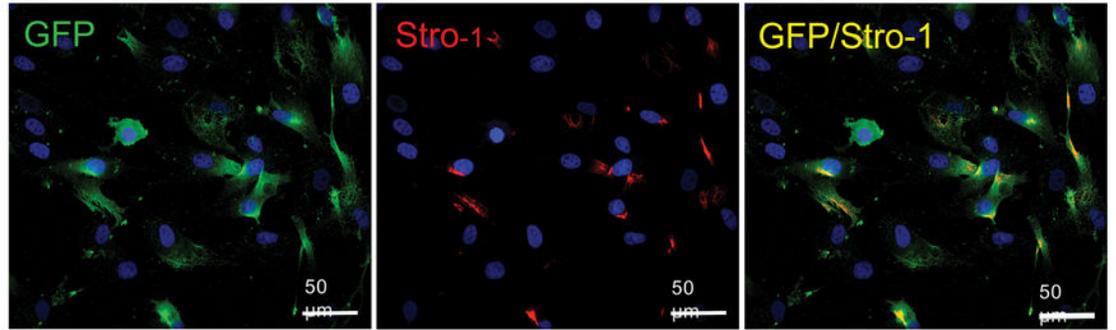
Figure 6. Adult SIPs are serially transplantable

(a) CD34+CD45+ cells isolated from bone marrow of primary SIPs recipients (labeled as "Donor Sh") were transplanted into NSG mice (n=3). Cells were gated based upon FSC, SSC, Ter119-, and 7AAD-. Positive control animals received CD34+CD117+ human mobilized peripheral blood stem cells; negative controls were non-transplanted animals. Percentages shown are based on the total number of human CD45+ cells in the plot. **(b)** Upper panel: SIPs remain in BM of transplanted animals. At 2.5 years post-transplant, BM from 2 of the secondary recipients that had received SIPs transduced with a lentivector

encoding GFP, were sorted based on Stro-1 and grown in culture. Human-derived Stro-1+, GFP+ cells were detected in culture. Images were acquired with a Zeiss Axiovert 200M at 20X magnification. DAPI (blue) labels all nuclei. Lower panel: PCR analysis, using human-specific primers for GAPDH demonstrated that SIPs sorted from primary recipients were of human origin. Samples were run simultaneously in the same gel, but the gel was cropped to improve clarity and show amplification of samples specific to cultured cells.
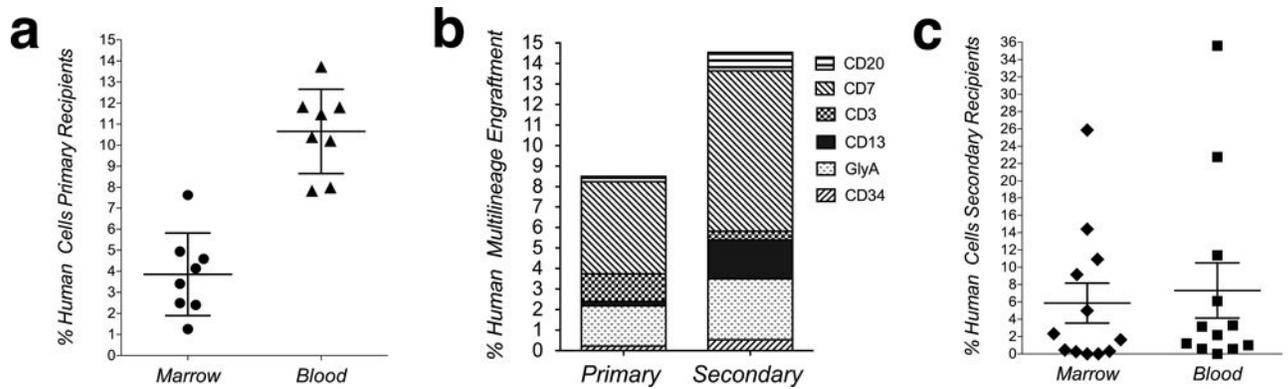
Figure 7. Clonally-derived SIPs convert to serially transplantable HSC after transplantation into fetal sheep

(a) Percentage of total human hematopoietic (myeloid and lymphoid) engraftment at 75 days post-transplantation in animals transplanted with clonally-derived SIPs (n=8); each mark represents levels of engraftment within one animal. **(b)** Clonally-derived SIPs generated HSC with multilineage differentiation capability in primary and secondary recipients; overall percentage of engraftment within the different hematopoietic lineages. **(c)** HSC generated from clonally-derived SIPs are serially transplantable; human hematopoietic engraftment in blood and marrow in secondary animals (n=12), as determined by the percentage of human myeloid and lymphoid cells at 2 months post-transplant.