Hematopoietic stem cells (HSCs) continuously replenish all blood cell lineages throughout their lifetime. Incipient hematopoiesis is first detected extraembryonically in the yolk sac and later in the aorta–gonad–mesonephros region, from where it moves transiently to the placenta and liver before being stabilized in the fetal BM (Wang and Wagers, 2011). In the adult stage, HSCs reside in a highly complex and dynamic microenvironment of the BM commonly referred to as the HSC niche (Schofield, 1978). The interactions between the niche constituents and HSCs ensure hematopoietic homeostasis by regulating HSC self-renewal, differentiation, and migration and by integrating neural and hormonal signals from the periphery (Méndez-Ferrer et al., 2009, 2010; Mercier et al., 2012). However, HSC maintenance and expansion ex vivo still remains challenging mainly because of our limited knowledge on the in vivo HSC niche constituents and the factors that drive HSC self-renewal.

The intermediate filament protein Nestin labels populations of stem/progenitor cells, including self-renewing mesenchymal stem cells (MSCs), a major constituent of the hematopoietic stem cell (HSC) niche. However, the intracellular location of Nestin prevents its use for prospective live cell isolation. Hence it is important to find surface markers specific for Nestin+ cells. In this study, we show that the expression of PDGFRα and CD51 among CD45- Ter119- CD31- mouse bone marrow (BM) stromal cells characterizes a large fraction of Nestin+ cells, containing most fibroblastic CFUs, mesospheres, and self-renewal capacity after transplantation. The PDGFRα+ CD51+ subset of Nestin+ cells is also enriched in major HSC maintenance genes, supporting the notion that niche activity co-segregates with MSC activity. Furthermore, we show that PDGFRα+ CD51+ cells in the human fetal BM represent a small subset of CD146+ cells expressing Nestin and enriched for MSC and HSC niche activities. Importantly, cultured human PDGFRα+ CD51+ nonadherent mesospheres can significantly expand multipotent hematopoietic progenitors able to engraft immunodeficient mice. These results thus indicate that the HSC niche is conserved between the murine and human species and suggest that highly purified nonadherent cultures of niche cells may represent a useful novel technology to culture human hematopoietic stem and progenitor cells.

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Nestin+ cells express high levels of HSC niche genes

Nestin+ cells express high levels of HSC maintenance genes such as the chemokine Cxcl12, Vcam1 (vascular cell adhesion molecule-1), Angpt1 (angiopoietin-1), S1f (stem cell factor), and Opn.
Figure 1. Mouse BM PDGFRα+ CD51+ cells constitute an enriched population of Nes-GFP+ cells. (A) Stromal (CD45− Ter119− CD31−) Nestin+ cells were isolated from Nes-GFP transgenic mice and analyzed for the expression of the indicated cell surface markers by flow cytometry. n = 3–9. (B) Expression of PDGFRα and Nes-GFP, CD51 and Nes-GFP, and Sca-1 and Nes-GFP markers on CD45− Ter119− CD31− stromal cells as determined by flow cytometry. (C) PDGFRα and CD51 expression on Nes-GFP+ BM cells. (D) Stromal PDGFRα+ CD51+ cells were analyzed for Nes-GFP expression by flow cytometry. Numbers in B–D indicate the percentage of positive cells present in the indicated gates. Data are representative of three to nine independent analyses with comparable results. (E) Stromal PDGFRα+ CD51+ cells were isolated from the BM of C57BL/6 wild-type mice, and Nestin mRNA levels were measured by real-time PCR. (F and G) Stromal BM cells from wild-type mice (F) and stromal Nestin+ cells from Nes-GFP transgenic mice (G) were sorted based on expression of PDGFRα and CD51 and the expression of the indicated genes assessed by real-time PCR. GFP+ cells from Nes-GFP transgenic mice were compared with BM cell populations in F. Data are from three to eight independent sorting experiments. *, P < 0.05; unpaired two-tailed Student’s t test. All error bars indicate SEM.
expression analysis showed that within the PDGFRα+ CD51+ population, a small fraction of Nes-GFP+ cells (~25%; Fig. 2 A, purple gate) also expressed meaningful levels of HSC niche genes, notably Opn and Scf (Fig. 2 C). All together, these results show that PDGFRα+ CD51+ stromal cells express the key HSC niche genes contained in Nestin+ cells and suggest that this population may represent a suitable alternative to prospectively isolate niche cells.

**PDGFRα+ CD51+ BM stromal cells recapitulate the MSC activity of Nestin+ cells**

Our previous studies have revealed that Nes-GFP+ cells contain all of the MSC activity in the BM, as determined by their exclusive ability to form CFU-Fs and mesospheres that can self-renew in vivo (Méndez-Ferrer et al., 2010). Because both MSC and HSC niche activities are very rare in BM, and likely are derived from a subset of Nes-GFP+ cells, it remains possible that the two activities are not conferred by the same cell. Having found that niche activity was enriched in PDGFRα+ CD51+ cells, which comprised 60% of Nes-GFP+ cells, we next tested whether MSC activity co-segregated with niche function. CFU-F assays of sorted double- and single-positive

Figure 2.

**Stromal PDGFRα+ CD51+ cells express high levels of HSC regulatory genes in contrast to the small fraction of PDGFRα− CD51− Nes-GFP+ cells.** (A) CD45− Ter119− CD31− stromal cells from Nes-GFP transgenic mice were gated as shown: PDGFRα+ CD51+ (middle; red), PDGFRα− CD51− (middle; gray), PDGFRα− CD51− Nes-GFP− (left; black), PDGFRα− CD51− Nes-GFP+ (left; blue), PDGFRα+ CD51+ Nes-GFP− (right; purple), and PDGFRα+ CD51+ Nes-GFP+ (right; green). (B and C) Stromal PDGFRα and CD51 BM cell populations were sorted according to the gates in A and analyzed for expression of the HSC regulatory genes Cxcl12, Vcam1, Angpt1, Opn, and Scf by real-time PCR analysis. n = 3 independent sorting experiments. *, P < 0.05; unpaired two-tailed Student’s t test. All error bars indicate SEM.
fractions revealed that mesenchymal progenitor activity was largely confined to the stromal PDGFRα+ CD51+ fraction (Fig. 3A). In addition, PDGFRα+ CD51+ cells, but not other stromal subpopulations, plated at clonal densities (<500 cells/cm²) or by single-cell FACS sorting deposition were able to form nonadherent primary spheres with the same efficiency as Nes-GFP+ cells (Fig. 3B). When dissociated, these spheres could be passaged and formed secondary spheres, demonstrating the in vitro self-renewal capacity of PDGFRα+ CD51+ cells (not depicted). In contrast, the rare and small spheres (<40 µm in diameter) forming from PDGFRα+ CD51− and PDGFRα− CD51+ subpopulations (Fig. 3B) did not have the capacity to form secondary spheres in culture. When PDGFRα+ CD51+ cells were isolated from Nes-Gfp mice, the majority of the clonal spheres (~40–130 µm in diameter) retained Nes-GFP expression until ~1.5 wk in culture (Fig. 3C). Using conventional adherent MSC culture conditions (Phinney et al., 1999; Pittenger et al., 1999), sorted PDGFRα+ CD51+...
cells rapidly down-regulated HSC maintenance gene expression along with Nes-GFP (not depicted).

Clonally expanded PDGFRα+ CD51+ spheres plated into in vitro mesenchymal lineage differentiation conditions exhibited robust tri-lineage potential, with up-regulation of osteoblastic (Fig. 3 D), adipocytic (Fig. 3 E), and chondrocytic (Fig. 3 F) differentiation genes during a 12–20-d period. Multilineage differentiation was confirmed by morphological and histochemical characterization of mature osteoblastic (Fig. 3 G), adipocytic (Fig. 3 H), and chondrocytic (Fig. 3 I) lineage phenotypes after >30 d in culture.

**Self-renewing murine PDGFRα+ CD51+ cells are able to transfer hematopoietic niche activity in vivo**

To examine whether PDGFRα+ CD51+ cells were capable to self-renew in vivo and transfer hematopoietic activity (Sacchetti et al., 2007; Méndez-Ferrer et al., 2010), we used two different transplantation approaches to deliver single clonal PDGFRα+ CD51+ spheres derived from Nes-Gfp mice. In the first approach, single spheres were incorporated into collagen grafts and implanted under recipients’ kidney capsules (Fig. 3 J and L), and alternatively, spheres were implanted s.c. within collagen gelfoam grafts (Fig. 3 K and M). 8 wk after transplantation, Nes-GFP+ cells were detected inside the grafts and in close contact with host CD45+ hematopoietic cells recruited in the extra-medullary microenvironment (Fig. 3, L and M). In contrast, PDGFRα− CD51+ and PDGFRα− CD51− spheres did not display any self-renewing Nes-GFP+ cells, and very few CD45+ cells were present inside the graft (not depicted). Controls included nontransplanted kidney capsules and grafts without stromal cells that only showed very rare CD45+ inflammatory cells.

To further determine whether the ectopic grafts loaded with mouse PDGFRα+ CD51+ cells could provide a functional niche for HSCs to engraft, we quantified by FACS analysis the total Nes-GFP+ population, LepR+ cells also represent a major fraction (77 ± 6%; Fig. 4 F) associated with the double-tomato expressing LepR+ cells. We intercrossed LepR-cre knock-in mice with Lsl-tomato reporter and Nes-Gfp mice to evaluate this possibility directly. Indeed, we found that BM LepR+ cells largely overlap with Nes-GFP+ (80 ± 7%; Fig. 4 F) and PDGFRα+ CD51+ populations (63 ± 8%; Fig. 4 G). Within the total Nes-GFP+ population, LepR+ cells also represent a major fraction (77 ± 6%; Fig. 4 F) associated with the double-positive PDGFRα+ CD51+ subset of Nestin+ cells (Fig. 4 H). In keeping with their overlap with Nestin+ and PDGFRα+ CD51+ cells, LepR+ cells also contained CFU-F activity (Fig. 4 I).

**PDGFRα and CD51 identify Nestin+ cells in the human fetal BM**

The identification of surface markers representing Nes-GFP+ cells gave an opportunity to investigate whether a similar stromal population was present in the human BM. A population of human Nestin+ cells with similar morphology to murine cells has indeed been observed in the human adult BM (Ferraro et al., 2011) and cultured adherent BM stromal cells (Schajnovitz et al., 2011). In keeping with these results, we observed in the developing human fetal BM the presence of elongated, peri-cyte-like Nestin+ cells organized in elongated logettes surrounded by forming cartilaginous bone (Fig. 5, A–F). Human Nestin+ cells were distinct from the vascular endothelial cells because they did not express VE-cadherin (Fig. 5 C); however, they showed a perivascular distribution in regions close to the bone (Fig. 5 F) or within the BM parenchyma in close contact with α-smooth muscle actin-positive vasculature (Fig. 5 D). Immunostaining for human hematopoietic lineage (Lin) markers (CD2, CD3, CD14, CD16, CD19, CD56, and CD235a),

Relationship between Nestin+, PDGFRα+ CD51+ MSCs, and other putative niche cells

A previous study has suggested that PDGFRα+ Sca-1+ (PαS) cells comprise MSCs in the BM (Morikawa et al., 2009). However, we found that Sca-1 was not expressed on the vast majority of Nestin+ cells (Figs. 1 A and 4, A and B), suggesting that MSC activity in the BM lay outside of the PαS population. We hypothesized that the discrepancy between these results could originate from how cells were harvested because Morikawa et al. (2009) and Houdhian et al. (2012) isolated stromal cells from crushed bones and discarded the BM fraction. Indeed, we detected a stromal Nes-GFP+ resident population in compact bone, of which ~30% expressed Sca-1. In bone, Nestin+ and PαS cells overlapped (~23% of PαS cells are Nestin+) and were enriched for CFU-F activity (Fig. 4, C–E). Interestingly, PDGFRα+ CD51+ markers also label 45 ± 5% of Nes-GFP+ cells in bone (not depicted); these results suggest that PDGFRα+ CD51+ cells (or Nestin+ cells) are distinct from PαS cells in the BM and that PαS population is enriched in MSC activity in bone but not in BM.

Recent data have indicated that perivascular cells expressing leptin receptor (LepR) are the main source of SCF in the BM and that these cells are distinct from Nestin+ cells (Ding et al., 2012). However, Nestin+ cells (Méndez-Ferrer et al., 2010) and PDGFRα+ CD51+ cells (Fig. 1 F) express high levels of Sf, suggesting some overlap between Nestin+ (PDGFRα+ CD51+) and LepR+ cells. We intercrossed LepR-cre knock-in mice with Lsl-tomato reporter and Nes-Gfp mice to evaluate this possibility directly. Indeed, we found that BM LepR+ cells largely overlap with Nes-GFP+ (80 ± 7%; Fig. 4 F) and PDGFRα+ CD51+ populations (63 ± 8%; Fig. 4 G). Within the total Nes-GFP+ population, LepR+ cells also represent a major fraction (77 ± 6%; Fig. 4 F) associated with the double-positive PDGFRα+ CD51+ subset of Nestin+ cells (Fig. 4 H). In keeping with their overlap with Nestin+ and PDGFRα+ CD51+ cells, LepR+ cells also contained CFU-F activity (Fig. 4 I).
CD38, and CD34 showed that Nestin+ cells are also in close contact with Lin- CD38- CD34+ HSC/progenitor cells in the fetal human BM (Fig. 5 E). Immunofluorescence analyses for PDGFRα and CD51 expression revealed colocalization with Nestin+ cells in the human fetal BM (Fig. 5 F) as in the mouse BM. In the developing human BM (15–20 gestation weeks [gw]), we found that the double-positive PDGFRα+ CD51+ cells comprised ~6.0 ± 1.6% of the stromal (CD45- CD235a- CD31-) population (Fig. 5, G and H). These PDGFRα+ CD51+ stromal cells were also present in the adult human BM, albeit at a lower frequency (<1%) than fetal BM (Fig. 5 H). Cell sorting of stromal cells expressing PDGFRα and/or CD51 revealed robust NESTIN expression in PDGFRα+ cells (Fig. 5 I). Freshly isolated human fetal PDGFRα+ CD51+ cells expressed high levels of HSC maintenance genes (CXCL12, VCAM1, ANGPT1, OPN, and SCF; Fig. 5 J), suggesting that PDGFRα and CD51 also define a stromal population with HSC niche activity in human BM.

Human fetal PDGFRα+ CD51+ cells are bona fide MSCs

To test whether PDGFRα+ CD51+ cells exhibit features of MSCs, we evaluated CFU-F content in double- and single-positive fractions and found that the highest clonogenic capacity was in the PDGFRα+ CD51+ subpopulation (Fig. 6 A). Furthermore, sorted human PDGFRα+ CD51+ cells were able to efficiently form nonadherent spheres in comparison with other stromal subpopulations (Fig. 6, B–D), when plated at clonal densities using the same condition as for the murine spheres. 1 wk after culture, human fetal and adult spheres continued to express PDGFRα, CD51, and NESTIN homogeneously, as seen by immunofluorescence analysis (Fig. 6 D). Human clonal PDGFRα+ CD51+ spheres were able to efficiently self-renew in vitro, forming secondary spheres upon dissociation that retain PDGFRα+ CD51+ expression in culture (not depicted). Clonally expanded fetal human PDGFRα+ CD51+ cells were also capable of robust tri-lineage differentiation into osteoblastic (Fig. 6, E and H), adipocytic (Fig. 6, F and I), and chondrocytic (Fig. 6, G and J) mesenchymal lineages, further demonstrating their MSC identity.

Human PDGFRα+ CD51+ BM cells are a small subset of CD146+ cells, enriched for MSC and niche activities

Because culture-expanded human CD146+ osteoprogenitor cells were previously shown to be highly enriched in CFU-F activity and capable of establishing the hematopoietic microenvironment in a xenotransplantation model (Sacchetti et al., 2007), we evaluated the relationship between stromal PDGFRα+ CD51+ and CD146-expressing cells. We found that stromal PDGFRα+ CD51+ spheres were able to efficiently self-renew in vitro, forming secondary spheres upon dissociation that retain PDGFRα+ CD51+ expression in culture (not depicted). Clonally expanded fetal human PDGFRα+ CD51+ cells were also capable of robust tri-lineage differentiation into osteoblastic (Fig. 6, E and H), adipocytic (Fig. 6, F and I), and chondrocytic (Fig. 6, G and J) mesenchymal lineages, further demonstrating their MSC identity.
Figure 5. Perivascular Nestin+ cells are present in the human fetal BM and express PDGFRα+ CD51+ cell surface markers. (A and B) Representative Toluidine Blue-stained section of a 17-gw human fetal femur showing a general view of the diaphyseal area (A) and details of the human fetal BM histological organization (B). Magnification of the boxed area in A is shown in B. Bar, 555 µm. (C and D) Immunofluorescence staining for NESTIN+ cells and VE-cadherin+ endothelial cells (C) or α-smooth muscle actin+ (α-SMA; D) in arterial vessels extending from the diaphysis toward the metaphysis region. Cell nuclei were stained with DAPI. Magnified images of the boxed areas are depicted in the insets. (E) Immunostaining for hematopoietic lineage (Lin) and CD38, CD34, and NESTIN cells in the human fetal BM. Human Lin− CD38− CD34+ HSCs/progenitor cells are indicated by arrowheads. (F) Human fetal BM cells were stained for NESTIN, PDGFRα, and CD51 expression by triple-immunofluorescence staining. Cell nuclei were stained with DAPI. (C and F) White dashed lines demarcate compact bone areas. (G) Representative flow cytometric profile of freshly isolated stromal (CD45− CD235a− CD31−) PDGFRα+ CD51+ cells in human 19-gw fetal BM. (H) Comparison of the PDGFRα+ CD51+ cell frequency in the stromal population between fetal and adult BM. (I and J) Stromal PDGFRα+ CD51+ cells were sorted from human fetal BM samples and NESTIN (I), and CXCL12, VCAM1, ANGPT1, OPN, and SCF mRNA levels were measured by real-time PCR (J). n = 4 independent experiments. *, P < 0.05; unpaired two-tailed Student’s t test. All error bars indicate SEM.
analysis of the fractionation of total CD146+ population in PDGFRα+ CD51+ (green gate) and non–double-positive cells (black gate) also confirmed that PDGFRα+ CD51+ cells were an enriched fraction for HSC maintenance genes within the CD146+. In addition, we have also observed that within the CD146+ population, most of the sphere-forming capacity was within the PDGFRα+ CD51+ subset (Fig. 7 D). These results indicate that PDGFRα+ CD51+ are a small subset of the CD146+ population that markedly enriches for HSC niche and MSC activities in the human fetal BM.

HSC niche activity of human fetal PDGFRα+ CD51+ cells
To assess in vivo self-renewal, single clonal human PDGFRα+ CD51+ spheres were culture-expanded and transplanted in conjunction with hydroxyapatite/tricalcium phosphate (HA/TCP) carrier particles s.c. into immunodeficient mice. Before transplantation, culture-expanded cells homogeneously expressed PDGFRα and CD51. 8 wk after transplantation, foci of murine hematopoietic activity could be detected inside the graft (Fig. 8 A). Because PDGFRα and CD51 epitopes are sensitive to degradation as a result of the decalcification process,
we evaluated the presence of MSCs in situ by staining for human-specific anti-NESTIN. Self-renewing Nestin+ cells were detected in the perivascular regions surrounding branching vessels containing murine Ter119+ red blood cells (Fig. 8, B–D). In addition, other constituents of the HSC stromal niche resembling the fetal BM microenvironment were also observed, including immature hyaline-like cartilage nodules, bone osteoid–like matrix containing osteoblast-like cells, and adipocytes (not depicted). Consistent with their self-renewal capacity, transplanted human PDGFRα+ CD51+ cells were capable of forming human secondary clonal spheres in culture (Fig. 8 E) that retained PDGFRα, CD51, and NESTIN expression (Fig. 8 F), as seen by immunofluorescence staining using human-specific anti-CD51 and anti-NESTIN antibodies.

To further determine whether the ectopic grafts loaded with human PDGFRα+ CD51+ cells could provide a functional niche for circulating recipient HSCs to engraft, we verified by FACS analysis the presence of phenotypic HSCs and progenitor cells. To ensure that the hematopoietic cells inside the ossicles were not derived from the human transplanted BM stromal cells but recruited from the recipient mice, all analyzed cells were gated on mouse CD45+ cells using a species-specific antibody. The frequency of CD45+ cells in the PDGFRα+ CD51+ ectopic grafts tended to be higher than in the control grafts (P = 0.104). However, the frequency of phenotypic hematopoietic progenitors (CD41− Lin− Sca1+ c-Kit+) and HSCs (CD41− Lin− Sca1+ c-Kit+ CD48− CD150+) in the PDGFRα+ CD51+ ectopic grafts was approximately eight- and sixfold higher in the PDGFRα+ CD51+ grafts than in the control group, respectively (Fig. 8 G). These results suggest that human PDGFRα+ CD51+ cells isolated from fetal BM are able to initiate ectopic HSC niche formation and recruit circulating HSCs and progenitors into the ectopic graft.
of LTC-ICs was increased by twofold when CD34+ cells were cultured with mesenspheres in comparison with CD34+ cells cultured with cytokines only (Fig. 9 C). Second, we analyzed the engraftment ability of ex vivo–expanded HSCs and progenitors. We found that mesensphere-expanded fetal BM CD34+ cells led to a significant increase in the proportion of engrafted NSG mice 8 wk after transplantation (80% vs. 9%; P < 0.05, Fisher’s exact test; Fig. 9 D). In contrast, there was a nonsignificant trend of enhanced engraftment in the group transplanted with cells cultured with cytokines only. Furthermore, mesensphere-expanded cells proved to have multilineage potential as they were able to differentiate along the myeloid and lymphoid lineages (Fig. 9 E). Collectively, these data demonstrate that PDGFRα+ CD51+ mesenspheres can efficiently expand a population enriched in HSPCs capable of multilineage engraftment.

HSPC expansion by PDGFRα+ CD51+ mesenspheres is contact independent

In the co-culture system, we observed that CD34+ cells tend to aggregate around the PDGFRα+ CD51+ mesenspheres (Fig. 10 A), raising the question of whether or not direct contact with the mesenspheres is necessary for the HSC/progenitor expansion. We therefore plated PDGFRα+ CD51+ mesenspheres either directly with CD34+ cells or in the upper chamber of a transwell unit with 0.4-µm pore polycarbonate membrane...
to prevent direct contact with CD34+ cells. Unexpectedly, we observed that direct contact between mesospheres and CD34+ cells was not required for the expansion of CD45+ cells, MPPs, and HSC-enriched population, quantified by flow cytometry after 10 d. Cells were also scored for CFU-C activity. \( n = 3 \). (C) Long-term HSCs were quantified from the input Lin− CD34+ population or after 10 d of co-culture with or without mesospheres using LTC-IC assay. \( n = 3 \). * \( P < 0.05 \); unpaired two-tailed Student’s \( t \) test. All error bars indicate SEM. (D) \( 2 \times 10^4 \) input CD34+ cells or a final culture equivalent to \( 2 \times 10^4 \) CD34+ starting cells cultured with or without mesospheres were transplanted into NSG mice, and human BM engraftment was evaluated 8 wk after transplantation. \( n = 10–11 \) mice per group. * \( P < 0.05 \); Fisher’s exact test; n.s., not significant. (E) multilineage human hematopoietic engraftment was evaluated by detection of myeloid (CD11b and CD33) and lymphoid (CD19) markers. Representative flow cytometry plots of BM cells from each experimental condition are shown.

to quantify the capacity of PDGFRα+ CD51+ mesospheres to expand HSCs, we then monitored cell expansion in function of the number of mesospheres per well. Although we observed a positive correlation between the number of spheres and CD45+ cells, the population enriched in HSC activity seems to be more sensitive to sphere numbers (Fig. 10 C). A minimum of 15–25 spheres/well was required to significantly

Figure 9. Fetal PDGFRα+ CD51+ mesospheres expand HSPCs ex vivo. (A) Gating strategy used to analyze hematopoietic populations by flow cytometry. (B) CD34+ cells were co-cultured with PDGFRα+ CD51+ mesospheres in serum-free media containing SCF, Flt3L, and TPO. CD45+ cells, MPPs, and HSC-enriched population were quantified by flow cytometry after 10 d. Cells were also scored for CFU-C activity. \( n = 3 \). (C) Long-term HSCs were quantified from the input Lin− CD34+ population or after 10 d of co-culture with or without mesospheres using LTC-IC assay. \( n = 3 \). * \( P < 0.05 \); unpaired two-tailed Student’s \( t \) test. All error bars indicate SEM. (D) \( 2 \times 10^4 \) input CD34+ cells or a final culture equivalent to \( 2 \times 10^4 \) CD34+ starting cells cultured with or without mesospheres were transplanted into NSG mice, and human BM engraftment was evaluated 8 wk after transplantation. \( n = 10–11 \) mice per group. * \( P < 0.05 \); Fisher’s exact test; n.s., not significant. (E) Multilineage human hematopoietic engraftment was evaluated by detection of myeloid (CD11b and CD33) and lymphoid (CD19) markers. Representative flow cytometry plots of BM cells from each experimental condition are shown.

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promote HSC expansion, and in contrast, plating >25 mesospheres/well negatively impacted the expansion of HSC-enriched population (Fig. 10 C). These results suggest that a very specific dosage of the factors secreted by PDGFRα+ CD51+ mesospheres is essential to expand HSPCs.

**Human PDGFRα+ CD51+ mesospheres express SCF and rescue HSPC expansion in the absence of SCF**

To gain some insight into the factors secreted by PDGFRα+ CD51+ mesospheres mediating their capacity to expand HSPCs, we cultured CD34+ cells with or without mesospheres in media containing different combinations of cytokines. When SCF was absent from the culture media, the HSC-enriched population was significantly reduced. In these culture conditions, the presence of the PDGFRα+ CD51+ mesospheres rescued HSPC expansion, yielding a 36- and 7.5-fold expansion, as compared with control media without mesospheres and day 0 input, respectively (Fig. 10 D). In the absence of Flt3L or TPO, PDGFRα+ CD51+ mesospheres also rescued HSC expansion, but the effect was not as marked. Indeed, PDGFRα+ CD51+ mesospheres generated a 3.7- and 9.4-fold expansion in the absence of Flt3L and TPO, respectively, in comparison with media without spheres (Fig. 10 D). The expansion of MPPs and total CD45+ cells was also rescued by PDGFRα+ CD51+ mesospheres in all culture conditions. Furthermore, gene expression analyses revealed that PDGFRα+ CD51+ mesospheres expressed high levels of SCF compared with total BM, whereas levels of Flt3L and TPO did not differ significantly (Fig. 10 E). Immunofluorescence analyses showed that PDGFRα+ CD51+ mesospheres homogeneously expressed both NESTIN and SCF (Fig. 10 F). Thus, these data suggest that Nestin+ PDGFRα+ CD51+ mesosphere-derived SCF is likely to play an important role in HSPC maintenance.

**DISCUSSION**

Although near homogeneous populations of HSCs and progenitors have been extensively isolated and characterized, the identity and role of the stromal cells regulating hematopoiesis remain largely undefined. Progress has been hampered by the limited availability of freshly isolated tissues and the paucity of selective stromal markers and genetic tools. Common methods to isolate human MSCs have widely relied on plastic adherence and in vitro expansion of adherent cells, which invariably lead to heterogeneous stromal populations whose biological and immunophenotypic properties are modulated in culture (Sacchetti et al., 2007; Delorme et al., 2008; Tanabe et al., 2008; Liu et al., 2012). Here, we have used Nes–Gfp transgenic mice that mark a highly enriched fraction of MSCs that form the HSC niche (Méndez-Ferrer et al., 2010) to identify an equivalent in situ population defined by PDGFRα+ CD51+ CD45− CD31− CD235a− (or Ter119− in mice) representing a subset of Nestin+ cells that can be isolated prospectively in both mouse and human BM.

Although our previous study has suggested that the two stem cell types of the BM formed a single niche, only a small fraction of Nestin+ cells exhibits MSC activity by mesosphere or CFU-F assays (Méndez-Ferrer et al., 2010), likely because of the combination of Nestin+ cell heterogeneity and harsh isolation protocols that may have altered cell viability. The fact that the frequency of Nestin+ cells (0.03–0.08%) in mouse BM is higher than that of HSCs left the possibility that MSC activity and HSC maintenance properties could be conferred by distinct cells. The present experiments have given more insight in this question as PDGFRα+ CD51+ stromal cells marked a subset (~60%) of Nestin+ cells that enriched similarly for both HSC niche and MSC activities compared with the remaining Nestin+ cells. These results lend further support to the idea that these two activities may co-segregate in the BM.

Our results show that PDGFRα, an early development marker of a transient wave of MSC progenitors derived from neuroepithelial and neural crest lineages (Takashima et al., 2007), is a major surface marker for Nestin+ MSCs. Because neural crest stem cell–derived spheres also express Nestin (Nagoshi et al., 2008), both markers may overlap during early development. Our data indeed predict a significant overlap between Nestin+ cells and a population of CD45− Tie-2− CD31− CD45+ CD31− cells isolated from embryonic day 15.5 mouse fetal bones capable of generating heterotopic BM niche in a transplantation model (Chan et al., 2009). Although PDGFRα was recently used to isolate CD45− Ter119− PaxS cells from the adult mouse bone that enriched for CFU-F activity and differentiation capability into mesenchymal lineages (Monikawa et al., 2009), our study suggests that the BM, Nestin+ and PaxS cells are distinct cell populations. The fact that a higher proportion of Nestin+ cells express Sca-1 in bone raises interesting questions about functional differences of bone- versus BM-derived MSCs that should be investigated in the future.

A major advance of the current study is the identification of a population similar to Nestin+ perivascular cells in the human BM, which lie in close contact with human HSCs/progenitor cells. In the human fetal BM, PDGFRα and CD51 mark a subset of stromal cells expressing Nestin that is highly enriched in CFU-F activity. Like its mouse counterpart, freshly sorted human stromal PDGFRα+ CD51+ cells also express high levels of HSC maintenance genes and efficiently form clonal multipotent self-renewing mesospheres. Importantly, these cells could heterotopically reconstitute a BM niche populated by recruited HSPCs, and containing a subset of self-renewing perivascular cells that retained NESTIN expression.

A previous study has shown that human CD146+ BM cells comprised osteoprogenitors capable of generating hematopoiesis in heterotopic bones (Sacchetti et al., 2007). Although our results indicate that CD146 is not expressed on murine Nestin+ cells, a genome-wide expression profile of these cells was closest to that of human CD146+ BM cells (Méndez-Ferrer et al., 2010), suggesting that human CD146 may mark a stromal cell similar to murine Nestin+ cells. Indeed, our results in the human system indicate that PDGFRα+ CD51+ cells comprise a restricted subset of CD146+ stromal cells further enriched for HSC niche and MSC activities in the fetal human BM.
shows that PDGFα+ CD51+ mesospheres expand a population containing phenotypically defined CD45+ Lin- CD34+ CD38- CD90+ CD49f+ long-term HSCs (Notta et al., 2011).

Ex vivo human HSC expansion still represents a challenge in part because of our limited knowledge on the in vivo HSC niche constituents and factors secreted by these cells. Our study shows that PDGFα+ CD51+ mesospheres expand a population containing phenotypically defined CD45+ Lin- CD34+ CD38- CD90+ CD49f+ long-term HSCs (Notta et al., 2011).
Although this set of markers was not validated after prolonged culture, we found that expansion of the CD45<sup>+</sup> Lin<sup>-</sup> CD34<sup>-</sup> CD38<sup>-</sup> CD90<sup>-</sup> CD49f<sup>+</sup> cell population correlated with both LTC-IC expansion and increased engraftment in NSG mice. Our study thus provides a novel three-dimensional co-culture system using PDGFRα<sup>+</sup> CD51<sup>+</sup> mesospheres that will likely prove to be a useful platform to identify niche components critical for HSC ex vivo maintenance and expansion.

We have explored herein the role of SCF because a recent study has suggested that it was produced by endothelial and LepR<sup>+</sup> perivascular cells that were distinct from Nestin<sup>+</sup> cells in mouse BM (Ding et al., 2012). However, Nestin<sup>+</sup> MSCs also express high levels of SCF (Méndez-Ferrer et al., 2010), and our system using PDGFRα<sup>+</sup> CD51<sup>+</sup> mesospheres that uniformly express Nestin and SCF have a direct impact on the ex vivo expansion of HSCs/progenitors and can rescue HSC maintenance in the absence of exogenous SCF. Altogether, these data strongly suggest that Nestin<sup>+</sup> cells may represent an essential source of SCF in the human BM.

In summary, our results demonstrate the existence of a self-renewing, multipotent population of Nestin<sup>+</sup> MSCs as an important constituent of the human fetal HSC niche. Thus, this study provides the groundwork for isolation of highly purified populations of MSCs that will shed important insight on the molecular mechanisms mediating HSC maintenance and expansion.

**MATERIALS AND METHODS**

**Mouse strains.** All murine experiments were performed using adult 8–12-wk-old animals. All mice were housed in specific pathogen–free facilities at the Albert Einstein College of Medicine (Einstein) animal facility, and all of the experimental procedures were approved by the Animal Care and Use Committee of Einstein. C57BL/6 and C57BL/6 Ly5.2 (CD45.1) mice were purchased from the National Cancer Institute (Frederick Cancer Research Center). B6.129-LepR<sup>+</sup>(GFP<sup>+</sup>)<sup>+</sup>/J (LepR<sup>+</sup>) and B6.Cg-Gt(ROSa)26Sort<sup>+</sup>MegE-iCre<sup>+</sup>/J (LSL-ttdTomato) mice were purchased from The Jackson Laboratory. Nes-GFP transgenic mice (Mignone et al., 2004) and NOD-scid Il2rg<sup>-/-</sup> (NSG) immunocompromised mice were bred and used at Einstein.

**Cell isolation.** BM primary cells were isolated as previously described (Méndez-Ferrer et al., 2010) with minor modifications. In brief, femora, tibia, and humeri BM were gently flushed in L-15 FACS buffer (Méndez-Ferrer et al., 2010) and after erythrocyte lysis, digested with 1 mg/ml collagenase IV (Sigma-Aldrich) in HBSS (Gibco) with 10% FBS (STEMCELL Technologies) for 30 min at 37°C. For flow cytometry sorting, cells were enriched by immunomagnetic depletion using anti-CD45 magnetic beads (Miltenyi Biotec) according to the manufacturer’s recommendations. Cells were sorted on a FACSAnia (BD) to >95% purity. Human fetal BM samples, between 13 and 20 gw, were obtained from the Einstein Human Fetal Tissue Repository by protocols approved by the Institutional Review Board. Human fresh adult BM samples were commercially obtained from Lonza.

**Flow cytometry.** Fluorescein-conjugated or biotinylated mAbs specific to mouse CD45 (clone 30-F11), CD45.1 (clone A20), Ter119 (clone Ter-119), PDGFRα (clone APA5), CD51 (clone RMV-7), CD44 (clone IM7), CD130 (clone KGP130), c-Kit (clone 2B8), CD135 (clone A2F10), CD90 (clone S3-2.1), CD34 (clone RAM34), CD166 (clone eBioALC48), Sca-1 (clone D7), CD41 (clone MWReg30), CD133 (clone 13A8), CD11b (clone M1/70), CD150 (clone TC15-12F12.2), CD61 (clone 2C9G3), hematopoietic lineage cocktail, and corresponding isotype controls were purchased from eBioscience. P75 (clone 2E3) was purchased from Abcam. CD10 (clone SNS/C-L4-1A1) was purchased from Santa Cruz Biotechnology, Inc. CD31 (clone MEC13.3), CD105 (clone MJ7/18), and CD48 (clone HM48-1) were from BioLegend, whereas CD29 (clone KM16) and CD146 (clone ME-9F1) were purchased from BD. Ng2 rabbit polyclonal was obtained from EMD Millipore. Secondary antibodies Alexa Fluor 633 goat anti–rabbit IgG, Alexa Fluor 633 goat anti–mouse IgG, and Alexa Fluor 633 goat anti–rat IgG were obtained from Molecular Probes. Fluorochrome-conjugated or biotinylated mAbs specific to human CD45 (clone 2D1), CD235a (clone HIR2), CD31 (clone WM59), hematopoietic lineage cocktail, CD38 (clone HB7), CD49f (clone eBioGoH3), CD90 (clone eBio5E10), CD34 (clone 4H11), CD11b (clone ICRF44), CD53 (clone WM-53), and CD19 (clone HB19) were obtained from Biolegend. PDGFRα (clone 6R1) and CD146 (clone PIH12) were purchased from BD, and finally CD51 (clone NR1-M9) was purchased from BioLegend. Nestin<sup>+</sup>–positive staining was gated in reference to cells from wild-type mice without the GFP transgene, and positive specific antibody labeling was gated in reference to corresponding isotype control or fluorescence-minus-one (FMO) corresponding sample. Multiparameter analyses of stained cell suspensions were performed on an LSRII (BD) and analyzed with FlowJo software (Tree Star). DAPI<sup>+</sup> cells were evaluated for all of the analyses.

**Cell culture and differentiation.** For clonal sphere formation, cells were plated at clonal density (<500 cells/cm<sup>2</sup>) or by single cell sorting into ultra-low adherent plates as previously described (Méndez-Ferrer et al., 2010). Cells were kept at 37°C with 5% CO<sub>2</sub> in a water-jacketed incubator and left untouched for 1 wk to prevent cell aggregation. One-half medium changes were performed weekly. All spheres in a given well were counted at day 9, and results are expressed as a percentage of plated cells.

For osteogenic, adipogenic, and chondrogenic differentiation, mouse or human PDGFRα<sup>+</sup> CD51<sup>+</sup> cells were treated with StemXVivo Osteogenic, Adipogenic, or Chondrogenic mouse- or human-specific differentiation media, according to the manufacturer’s instructions (R&D Systems). All cultures were maintained with 5% CO<sub>2</sub> in a water-jacketed incubator at 37°C. At specific time points, cells were collected for RNA or cytochemistry analysis. Osteogenic differentiation indicated by mineralization of extracellular matrix and calcium deposits was revealed by Alizarin Red S staining. Cells were fixed with 4% paraformaldehyde (PFA) for 30 min. After rinsing in distilled water, cells were stained with 40 mM Alizarin Red S (Sigma-Aldrich) solution at pH 4.2, rinsed in distilled water, and washed in Tris-buffered saline for 15 min to remove nonspecific staining. Adipocytes were identified by the typical production of lipid droplets. Chondrocytes were revealed by Toluidine Blue staining, which detects the synthesis of glycosaminoglycans. Cells were fixed with 4% PFA for 60 min, embedded in paraffin, and sectioned. Sections were incubated with 0.5% Toluidine Blue (Sigma-Aldrich) in distilled water for 15 min. To remove nonspecific staining, sections were rinsed thrice with running water (5 min each).

**CFU-F assay.** 1–3 × 10<sup>5</sup> mouse sorted cells were seeded per well in a 12-well adherent tissue culture plate using phenol red–free α-MEM (Gibco) supplemented with 20% FBS (HyClone), 10% MesenCult stimulatory supplement (STEMCELL Technologies), and 0.5% penicillin-streptomycin. One half of the media was replaced after 7 d and at day 14 cells were stained with Giemsa staining solution (EMD Chemicals). Human fetal BM cells were plated at 0.5–1 × 10<sup>5</sup> cells/well into 6-well adherent tissue culture plates using phenol red-free α-MEM with 20% FBS (STEMCELL Technologies) and 0.5% penicillin-streptomycin. One half of the media was replaced after 5 d, and at day 10 cells were stained and adherent colonies counted.

**RNA isolation and quantitative real-time PCR.** Sorted or cultured cells were collected in lysis buffer, and RNA isolation was performed using the Dynabeads mRNA DIRECT Micro kit (Invitrogen). Reverse transcription was performed using the RNA to cDNA EcoDry Premix system (Takara Bio Inc.) according to the manufacturer’s recommendations. Quantitative real-time PCR was performed as previously described (Méndez-Ferrer et al., 2010).
The relative mRNA abundance was calculated using the ΔCt method and multiplied by 100. Gene expression data were normalized to Gapdh. Human and mouse primer sequences are included in Table S1.

**Immunofluorescence staining.** Human fetal bones and HA/TCP grafts were fixed with 4% PFA for 2 h at 4°C, partially decalcified with 0.25 M EDTA for 2–3 d, and cryoprotected with 15–30% sucrose. Samples were then processed as described previously (Kawamoto, 2003) and immunostained using standard technique. Mesospheres, collagen, and gelfoam grafts were also processed as described above without the decalcification step and using Superfrost Plus slides (Thermo Fisher Scientific). The following antibodies were used as primary: Alexa Fluor 488 anti-GFP (Molecular Probes), anti–mouse CD45-PE (clone 30-F11; eBioscience), anti–mouse Ter119-PE (clone Ter119; eBioscience), anti–human REST (clone 19608 [R&D Systems] and N5413 [Sigma-Aldrich]), anti–human PDGFRα (clone C-20; Santa Cruz Biotechnology, Inc.), anti–human CD51-FITC (clone NK1-M9; BioLegend), anti–human VE-cadherin (clone 16B1; eBioscience), anti–human α-SMA–Cy3 (clone 1A4; Sigma-Aldrich), anti–human lineage-APC cocktail (eBioscience), anti–human CD38 (clone HIT2; eBioscience), and anti–human CD34-FITC (clone AC136; Miltenyi Biotec). The secondary antibodies used were Alexa Fluor 633 goat anti–mouse IgG, Alexa Fluor 568 goat anti–rabbit IgG, and Alexa Fluor 488 goat anti–mouse IgG (Molecular Probes). For nuclear staining, samples were treated with DAPI (Sigma-Aldrich). Images were captured using an Axio Examiner D1 confocal microscope (Carl Zeiss), and images were processed using the SlideBook software (Intelligent Imaging Innovations). We processed human fetal bones for Toluidine Blue staining as described previously (Kawamoto, 2003).

**In vivo transplantation.** For renal capsule collagen graft, five thousand freshly sorted cells or single spheres were gently resuspended in 15 µl of a collagen (BD) mixed with 2% of 1N NaOH and 10% of 10X PBS. The cell/collagen mix was then gently deposited into a 6-well plate and incubated at 37°C for 30 min to allow the collagen to solidify. Collagen grafts were then transplanted under the renal capsule of 8–12-wk-old anaesthetized mice. After 8 wk, kidneys/graffs were collected and processed for immunofluorescence and FACS analyses.

For s.c. gelfoam graft, transplantations were performed as previously described (Bianco et al., 2006) with minor alterations. Five thousand freshly sorted cells or single spheres were gently resuspended in 50 µl of sphere media. 5-mm³ cubes of sterile collagen sponges (Gelfoam; Pfizer) were hydrated into sphere media and then squeezed to remove air bubbles and allow the sponge to regain its size. Just before transplantation, sponges were blotted dry, and images were processed as described above without the decalcification step and stained using standard technique. Mesospheres, collagen, and gelfoam grafts were then processed as described previously without the decalcification step and using Superfrost Plus slides (Thermo Fisher Scientific). The following antibodies were used as primary: Alexa Fluor 488 anti-GFP (Molecular Probes), anti–mouse CD45-PE (clone 30-F11; eBioscience), anti–mouse Ter119-PE (clone Ter119; eBioscience), anti–human REST (clone 19608 [R&D Systems] and N5413 [Sigma-Aldrich]), and anti–human PDGFRα (clone C-20; Santa Cruz Biotechnology, Inc.). The secondary antibodies used were Alexa Fluor 633 goat anti–mouse IgG, Alexa Fluor 568 goat anti–rabbit IgG, and Alexa Fluor 488 goat anti–mouse IgG (Molecular Probes). For nuclear staining, sponges were treated with DAPI (Sigma-Aldrich). Images were captured using an Axio Examiner D1 confocal microscope (Carl Zeiss), and images were processed using the SlideBook software (Intelligent Imaging Innovations). We processed human fetal bones for Toluidine Blue staining as described previously (Kawamoto, 2003).

**Transplantation into NSG mice.** 2 × 10³ fresh human CD34+ cells or a final culture equivalent to 2 × 10³ CD34+ input cells cultured with or without mesospheres were transplanted via the retroorbital route in NSG mice. NSG mice were sublethally irradiated (200 cGy) at least 4 h before transplantation. BM engraftment was analyzed 8 wk after transplantation by FACS. Mice were scored as engrafted when transplanted human cells reconstituted both myeloid and lymphoid lineages. Significance was calculated according to the Fisher’s exact test.

**Online supplement material.** Tables S1 shows primer sequences used for mouse and human quantitative real-time PCR analyses. Online supplement material is available at http://www.jem.org/cgi/content/full/jem.20122252/DC1.

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