Cell

Self-Renewing Osteoprogenitors in Bone Marrow Sinusoids Can Organize a Hematopoietic Microenvironment

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SUMMARY

The identity of cells that establish the hematopoietic microenvironment (HME) in human bone marrow (BM), and of clonogenic skeletal progenitors found in BM stroma, has long remained elusive. We show that MCAM/CD146-expressing, subendothelial cells in human BM stroma are capable of transferring, upon transplantation, the HME to heterotopic sites, coincident with the establishment of identical subendothelial cells within a miniature bone organ. Establishment of subendothelial stromal cells in developing heterotopic BM in vivo occurs via specific, dynamic interactions with developing sinusoids. Subendothelial stromal cells residing on the sinusoidal wall are major producers of Angiopoietin-1 (a pivotal molecule of the HSC "niche" involved in vascular remodeling). Our data reveal the functional relationships between establishment of the HME in vivo, establishment of skeletal progenitors in BM sinusoids, and angiogenesis.

INTRODUCTION

Bone marrow (BM) is the only permanent hematopoietic organ in humans. During organogenesis, hematopoiesis is established through interaction of blood-borne hematopoietic stem cells (HSCs) with a local stroma of mesenchymal lineage established during ossification (reviewed in Bianco and Robey, 2000). The critical role of BM stroma for homing and long-term maintenance of hematopoiesis in mammalian bone was classically demonstrated by transfer of the hematopoietic microenvironment (HME) to an ectopic site upon in vivo transplantation of either BM fragments or BM stromal cells (BMSCs; Friedenstein et al., 1974; Maniatis et al., 1971; Tavassoli and Crosby, 1968). Concurrent formation of heterotopic bone in the same systems first revealed that progenitors of skeletal tissues (bone, cartilage, fibrous tissue, adipocytes) are included in postnatal BM stroma (Friedenstein, 1990), a notion that later evolved into the current concept that skeletal stem cells (SSCs [Bianco and Robey, 2004], also known as stromal or "mesenchymal" stem cells) coexist with HSCs in BM. However, the identity of cells that establish the heterotopic HME, as well as that of putative SSCs, has remained elusive. Anatomically, BM stroma includes virtually all nonhematopoietic cells types found in the BM microenvironment (osteoblasts, endothelial cells, fibroblasts, reticular cells [Weiss, 1976]), each of which, in principle, may play a critical role in hematopoiesis. For example, the establishment of an HSC "niche" (Moore and Lemischka, 2006) in the mouse BM has been ascribed to osteoblasts residing on bone surfaces (Arai et al., 2004; Calvi et al., 2003; Zhang et al., 2003), as well as to endothelial cells lining sinusoids (Kiel et al., 2005), suggesting a potential multiplicity of physiologically important microenvironments.

Establishment of hematopoiesis within heterotopic ossicles formed by transplantation of BM stroma reflects the interaction of circulating HSCs and progenitors of the host with a locally established HME. Therefore, transplantation of BMSCs represents an appealing model of BM organogenesis in which to identify HME-establishing cells. The value of the system has been limited, however,

by lack of markers suitable for visualization of specific stromal cell types other than mature osteoblasts. Whereas the donor origin of mature osteoblasts and the host origin of hematopoietic cells colonizing the "ossicle" have been directly proven (Krebsbach et al., 1997; Kuznetsov et al., 1997), the host origin of the endothelial cells has only been surmised, and donor or host origin of stromal cells proper (e.g., reticular cells) has never been demonstrated.

Similar limitations have prevented definition of the precise identity and properties of clonogenic progenitors found in BM stroma. Whereas the multipotency of a fraction of human skeletal progenitors has been demonstrated (Kuznetsov et al., 1997), the ability to self-renew has not been formally determined for any subset of stromal cells, detracting from the very claim of their "stemness" and at odds with the general popularity gained by the concept of "mesenchymal" stem cells. Self-renewal implies reconstitution of the same compartment of phenotypically defined progenitor cells in vivo. As applied to stromal progenitor cells, this postulates that (1) the identity/phenotype of explanted progenitor cells is defined and (2) cells with identical phenotype and properties are demonstrated within the tissues formed de novo following in vivo transplantation. Whereas multiple markers expressed in clonogenic stromal cells from human BM have been proposed (Barry et al., 1999; Deschaseaux and Charbord, 2000; Gronthos et al., 1999; Shi and Gronthos, 2003; Simmons and Torok-Storb, 1991; Vogel et al., 2003; Zannettino et al., 2003), none of them have been used (and most are not suitable) for in situ identification of cells prior to explanation and after in vivo transplantation.

In this study, we show that a specific phenotype defines stromal progenitors in human BM that regenerate bone and stroma and establish the HME in vivo. Marked by high expression of melanoma-associated cell adhesion molecule, MCAM/CD146 (Shih, 1999), this phenotype is shared by subendothelial cells of BM sinusoids in situ and is not duplicated by osteoblastic cells capable of forming heterotopic bone, but not an HME, in vivo. By following the fate of transplanted, human $CD146^+$ cells during organogenesis of heterotopic BM, we document their stepwise regeneration into $CD146^+$ subendothelial cells in de novo formed BM, from which human $CD146^+$ clonogenic cells can ultimately be isolated in culture. Our data suggest self-renewal of $CD146^+$ osteoprogenitors in vivo as an integral part of angiogenic events whereby sinusoids are established in vivo prior to hematopoiesis. Consistent with their nature as subendothelial cells, $CD146^+$ osteoprogenitors, but not their differentiated osteoblastic progeny, express Angiopoietin-1 (Ang-1), a pivotal regulator both of vascular remodeling (Suri et al., 1996) and of the HSC niche (Arai et al., 2004). Our data anatomically and functionally identify clonogenic skeletal progenitors with the capacity to self-renew, reveal their identity as cells that transfer the HME in vivo, and link their establishment and regeneration in BM to angiogenesis.

RESULTS

BMSCs, But Not Other Osteogenic Cell Strains, Transfer the HME In Vivo

The ability to establish an HME was evaluated by transplantation of cell strains derived from BMSCs from normal hematopoietic BM, human trabecular bone cells (HTBs), periosteal cells (PCs), fibrotic bone marrow of fibrous dysplasia of bone (FD; OMIM#174800), and as controls, muscle fibroblasts (MFs) and skin fibroblasts (SFs). By in vitro differentiation assays commonly used to characterize "mesenchymal" stem cells, all strains (except SFs) generated adipocyte-like cells or "mineralization nodules" (data not shown). When transplanted subcutaneously into immunocompromised mice, control cell strains (MFs and SFs) failed to generate either bone or BM (12/12 transplants), while bone-derived cell strains (HTBs and PCs) generated bone but no heterotopic BM (12/12 transplants each strain). As previously reported (Bianco et al., 1998), FD cells, derived from fibrotic, nonhematopoietic BM, also formed bone but not BM (data not shown). Only BMSCs derived from hematopoietic BM established both bone and hematopoietic tissue (12/12 transplants) (Figure 1A).

CD146 Expression Distinguishes BMSCs from Other Osteogenic Strains

Characterization of the same cell strains using a panel of markers (Table S1), including putative markers of "mesenchymal" stem cells (CD49a, CD63, CD90, CD105, CD140b, CD146, STRO-1, and alkaline phosphatase [ALP]), revealed that high levels of CD63 distinguished cell strains endowed with osteogenic potential in vivo (BMSCs, HTBs, PCs, and FD cells) from nonosteogenic MFs and SFs. Only a characteristic "high/bright" expression of CD146 distinguished BMSCs from all other (osteogenic and nonosteogenic) strains, which, in contrast, were all noted for a "low/dim" expression of CD146 (Figures 1B and S1A).

All BM Colony Forming Unit-Fibroblasts and Their Clonal Progeny, But Not All BM "Stromal" Cells, Express CD146

Primary cultures of BMSCs were established either as nonclonal (high density, $> 1.6 \times 10^5$ cells/cm^2) cultures or by plating BM cell suspensions at clonal density in order to obtain discrete colonies (colony forming unit-fibroblast [CFU-F] cultures) (Friedenstein, 1980). Randomly selected colonies (n = 22) were individually harvested from primary CFU-F cultures from two donors. Multiclonal strains (n = 3) were generated by pooling all primary colonies obtained in CFU-F cultures from three donors (29, 13, and 11 colonies, respectively). CD146 was expressed at high levels in ~99% of cells in 22/22 clones and in 3/3 multiclonal strains (Figure 2A). In contrast, only ~30% of the cells were $CD146^+$ in 3 nonclonal cultures (Figures 2A and S1B). FACS analysis revealed that high numbers of $CD146^-CD105^+$ or $CD146^-ALP^+$ cells (putatively mature

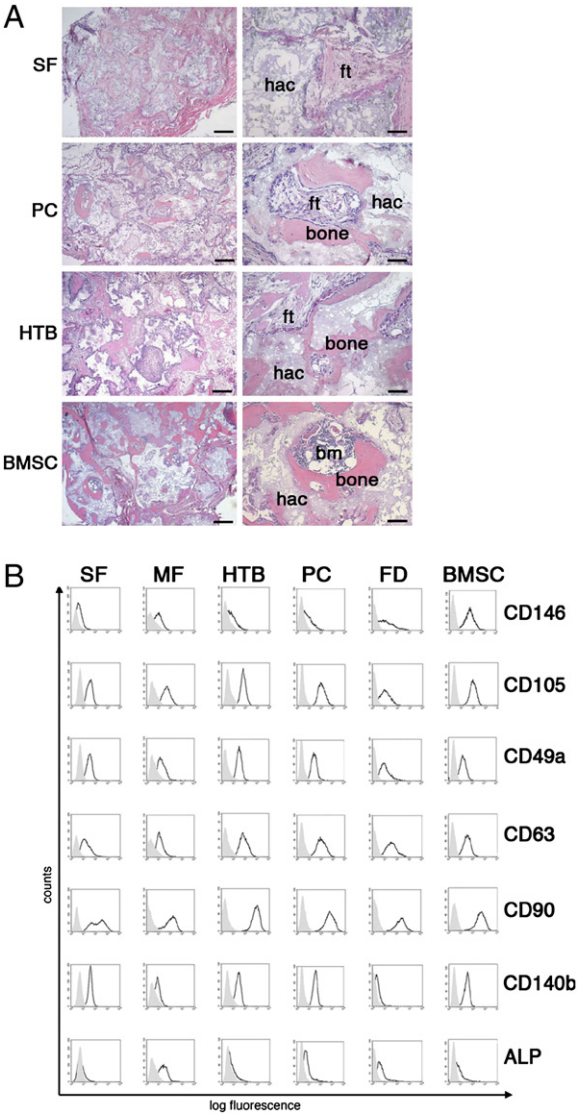

Figure 1. High Expression of CD146 Distinguishes BMSC, Capable of Establishing the HME, from Other Osteogenic or Nonosteogenic Cell Populations

(A) Histology of transplants of human skin fibroblasts (SFs), periosteal cells (PCs), human trabecular bone cells (HTBs), and bone marrow stromal cells (BMSCs) harvested at 8 weeks. Neither bone nor BM formed in transplants of SFs. Bone, but not marrow, formed in transplants of PCs and HTBs. Both bone and marrow formed in transplants of BMSCs. hac, hydroxyapatite carrier; bm, bone marrow; ft, fibrous tissue, H&E.

(B) FACS analysis of "mesenchymal stem cell" markers in cultures of SFs, MFs, HTBs, PCs, FD cells, and BMSCs grown under identical conditions up to passage 3. $CD146^{high/bright}$ expression distinguishes BMSCs from all other strains ($CD146^{low/dim}$). No other single marker is expressed differentially in BMSCs compared to all other strains.

osteoblastic cells) were included in nonclonal cultures (data not shown). Hence, establishing CFU-F cultures from unfractionated BM, while selecting for clonogenic cells, also selected for $CD146^+$ cells at the same time. These cells initiate the CFU-F cultures from which $CD146^{high/bright}$ BMSC strains are generated.

All CFU-Fs Are Found, and Most Are Recovered, in the $CD45^-CD146^+$ Fraction of BMNCs

By FACS analysis, $CD146^+$ cells accounted for 0.11% ± 0.02% of total human BM nucleated cells (BMNCs). Consistent with the known expression of CD146 in certain hematopoietic cells (e.g., T cells; Pickl et al., 1997), ~20% of $CD146^+$ cells coexpressed CD45 (not shown). $CD45^-$ cells within the BMNC suspensions were therefore magnetically separated prior to immunoselection of $CD146^+$ cells. $CD146^+$ cells were enriched ~10-fold (1.2% ± 0.9%) in the $CD45^-$ fraction compared to BMNCs. By dual-label FACS analysis (Figure 2B), $CD45^-CD146^+$ cells coexpressed ALP (78%–85%) and CD105 (>95%). In contrast, only ~2% of $CD45^-CD146^+$ cells coexpressed CD34, suggesting that >95% of $CD45^-CD146^+$ cells in human BM are not endothelial cells (ECs). $CD45^-$ cells were separated into $CD146^+$ and $CD146^-$ fractions by FACS, and colony forming efficiency (CFE) assays were conducted on both fractions (Figure 2C, Tables S2 and S3). An average of 2.6 ± 0.8 CFU-Fs/10^2 cells were observed in the $CD146^+$ fraction, which translates into an enrichment of 8.3 × 10^2 compared to BMNCs and a recovery of ~80% of the total CFU-Fs found in unfractionated BM (Table S3). No colonies were formed by $CD45^-CD146^-$ cells plated at the same or higher density (Figure 2C, Tables S2 and S3). When sorted and subjected to CFE assays, total $CD34^+$ cells and the $CD146^+CD34^+$ fraction failed to generate fibroblastic colonies (Table S4).

Adventitial Reticular Cells Are the In Situ Counterpart of $CD146^+$ CFU-Fs in Human BM

In human BM in situ, no labeling for CD146 was observed in erythroid or myeloid cells, megakaryocytes, endothelial cells, adipocytes, osteoblasts, osteocytes, or endosteal cells. Within hematopoietic tissue, CD146 labeling was restricted to adventitial reticular cells (ARCs; Bianco and Boyde, 1993; Tavassoli and Friedenstein, 1983; Weiss, 1976; Westen and Bainton, 1979). ARCs formed a subendothelial (adventitial) layer in sinusoidal walls, and projected reticular processes that associated with hematopoietic cells, away from sinusoidal walls (Figure 2D). Like the $CD146^+$ CFU-Fs from human BM, $CD146^+$ ARCs coexpressed endoglin (CD105) and ALP (both of which were also expressed by additional cell types in BM) in situ, but not CD34, CD31, or CD45 (not shown).

Cultured $CD146^+$ Cells Display Mural Cell Properties

Undifferentiated $CD146^+$ BMSCs expressed transcripts of early osteogenic progenitors but not of mature osteoblasts. Of note, HSC niche-related transcripts (Jagged-1, N-cadherin, CXCL12 [Sugiyama et al., 2006], and SCF [Duncan et al., 2005; Nagasawa et al., 1996; Zhang et al., 2003]) were highly expressed (Figure S2). Consistent with

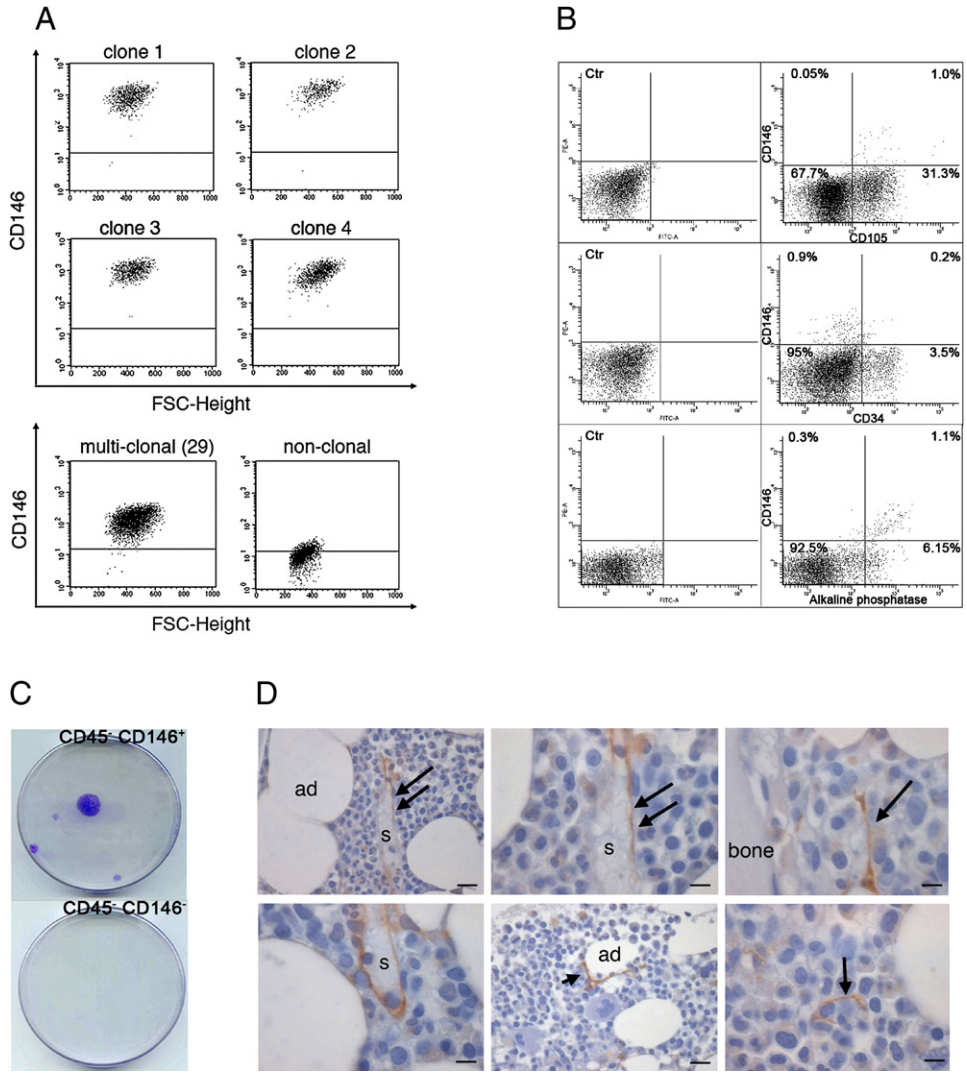

Figure 2. CD146$^+$ Stromal Cells Are the CFU-Fs and Derive from Adventitial Reticular Cells

(A) FACS analyses of randomly selected clones, formed in primary CFU-F cultures (4 clones representative of 22). CD146 is highly expressed in virtually all cells in each clone. Representative FACS analyses of multiclonal (29 pooled colonies) and nonclonal primary cultures of BMSCs. Whereas CD146 is highly expressed in virtually all cells in a multiclonal strain, only ~30% of cells are CD146$^+$ in a nonclonal primary culture.

(B) Dual-label FACS analyses of magnetically separated CD45$^-$ marrow cells. CD45$^-$CD146$^+$ cells coexpress CD105 (~99%) and alkaline phosphatase (ALP; ~80%). Only a minor fraction coexpresses CD34.

(C) CD146$^+$ and CD146$^-$ fractions within CD45$^-$ cells were separated by FACS. One hundred cells were plated in 100 mm dishes (1.6 cells/cm^2). Clonogenic cells were found only in the CD45$^-$CD146$^+$ fraction.

(D) Sections of human bone marrow immunolabeled for CD146 counterstained with hematoxylin. Labeling is restricted to adventitial cells of sinusoids (double arrows), extending "reticular" processes (single arrows) among hematopoietic cells.

their origin from ARCs, cultured CD146$^+$ cells failed to express endothelial markers or to differentiate into ECs when exposed to specific conditions. In contrast, they expressed a host of markers of subendothelial cells (mural cells/pericytes, MC [Jain, 2003], such as α-smooth muscle actin, NG2, calponin 1 and 3, PDGFRβ; Figure S2). Factors known to regulate mural cell growth and phenotype during the maturation phase of angiogenesis (Hirschi and D'Amore, 1996; Jain, 2003), such as FGF-2 and TGF-β1, modulated the mural cell phenotype and the proliferation of CD146$^+$ cells, in an opposite fashion (Figure S3). Whereas FGF-2 stimulated the proliferation of CD146$^+$ cells, TGF-β1 inhibited it, consistent with the effects of these factors on mural cells (Hirschi and D'Amore, 1996; Jain, 2003). When cocultured with endothelial cells, CD146$^+$ stromal cells behaved like mural cells; i.e., they directed the assembly and timed remodeling of pseudovascular structures in vitro, in sharp contrast with control, nondescript fibroblasts or unrelated cell types (Figure S4).

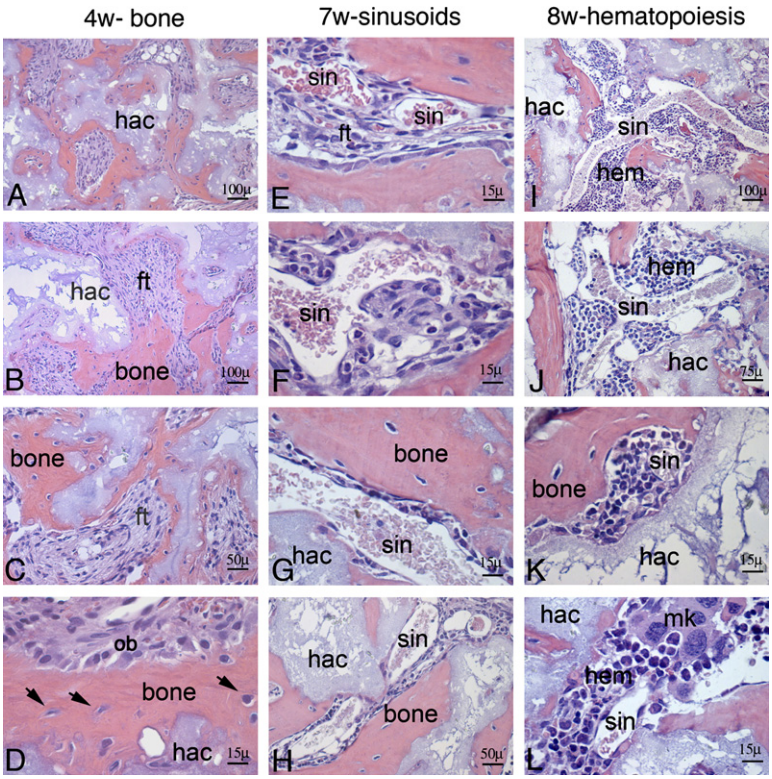

Figure 3. Development of Heterotopic Bone and BM in Transplants of BMSCs

(A–D) 4 weeks post-transplantation. Abundant new bone has formed along with mesenchymal fibroblastic tissue (ft in B and C). No hematopoiesis is detectable. Bone contains fully differentiated osteoblasts (D, ob) and osteocytes (D, arrows).

(E–H) 7 weeks post-transplantation. Large caliber, branching sinusoids have formed within the fibroblastic tissue.

(I–L) 8weeks post-transplantation. Hematopoietic cell clusters (hem) are obvious around sinusoids. hac, hydroxyapatite carrier; mk, megakaryocytes. H&E.

CD146$^+$ Cells form Bone and Hematopoiesis-Associated Human Stromal Cells In Vivo

We next asked if CD146$^+$ clonogenic cells could give rise to hematopoiesis-associated human stromal cells in vivo. Three BMSC strains, initiated by 11, 13, and 17 CD146$^+$ CFU-Fs, respectively, were culture-expanded and transplanted subcutaneously into immunocompromised mice. Analysis of transplants harvested at 4, 7, and 8 weeks revealed a defined developmental sequence in which bone formation preceded the appearance of a sinusoidal system, and ultimately of hematopoiesis. At 4 weeks, abundant human bone was associated with a hematopoiesis-free, human fibroblastic tissue (Figures 3A–3D and 4A–4D), noted for high expression of FGF-2 (Figure S5). Between 4 and 7 weeks, capillary-type vessels permeating this tissue remodeled into a system of large, branching sinusoids (Figures 3E–3H), reminiscent of those found in BM in situ. The endothelium of sinusoids was murine, and adventitial cells were human (Figures 4E–4H) and were established prior to appearance of hematopoiesis. Active TGF-β1 was localized to the developing sinusoidal wall (Figure S5). Murine hematopoietic tissue colonized the ossicle by 8 weeks (Figures 3I–3L). At this time, human stromal cells appeared as ARCs in the sinusoidal wall (Figures 4I–4L).

CD146$^+$ ARCs Regenerate CD146$^+$ ARCs In Vivo

Prior to transplantation, each cell strain homogeneously (~100%) expressed CD146 at high levels (Figure 5A). Four weeks post-transplantation, only ~3% of the human cells retained expression of CD146, all of which were physically associated with blood vessel walls in vivo (Figures 5B and S6). Differentiated human osteoblasts on bone and human fibroblastic tissue associated with bone did not express CD146. As sinusoids developed, CD146$^+$ cells remained associated with their walls and elongated over their abluminal surfaces (Figures 5C and S6). Once hematopoiesis was established by 8 weeks, human CD146$^+$ cells demonstrated exactly the same adventitial position, reticular morphology, and physical association with hematopoietic cells as the CD146$^+$ ARCs observed in human BM in situ (Figures 5C and S6). No CD146 immunoreactivity was observed in heterotopic ossicles devoid of hematopoiesis formed by transplanted human HTBs or PCs (data not shown).

Having determined that CD146$^+$ cells establish bone and human CD146$^+$ stromal cells at heterotopic sites, we asked if single CD146$^+$ CFU-Fs could generate both bone and hematopoiesis-supporting stroma. Four clonal strains, each derived from a single CFU-F, were isolated as described (Bianco et al., 1998; Kuznetsov et al., 1997), separately expanded through 20 population doublings in culture, and transplanted to generate heterotopic ossicles for histological study. At 8 weeks, complete ossicles, including bone, sinusoids, adipocytes, and CD146$^+$ ARCs (Figure 5D, Table S5), were generated by 2/4 clones, providing evidence for the ability of a single CD146$^+$ cell to establish both heterotopic bone and the HME.

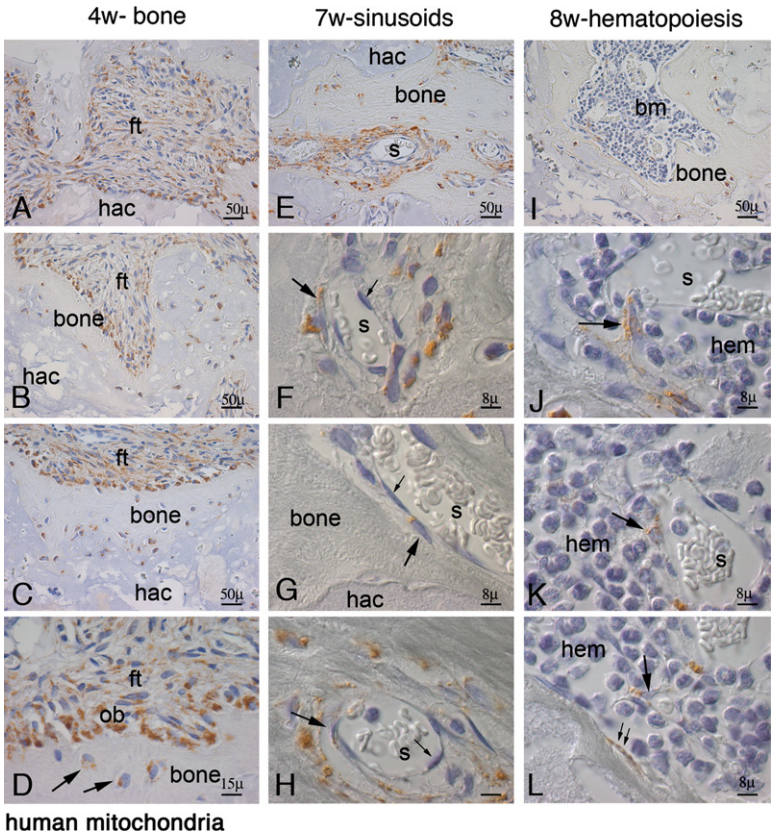

Figure 4. Development of Human Stromal Cells in the Chimeric Heterotopic BM—Immunolabeling for Human Mitochondria

(A–D) 4 weeks post-transplantation. The fibroblastic tissue (ft), bone-forming osteoblasts on bone surfaces (D, ob), and osteocytes within newly formed bone (D, arrows) are human.

(E–H) 7 weeks post-transplantation. ECs lining sinusoids (s) are murine (F–H, small arrows), whereas adventitial cells are human (F–H, large arrows).

(I–L) 8 weeks post-transplantation. Osteocytes within bone are human. Hematopoietic cells in newly formed marrow spaces (hem) and ECs lining sinusoids (J–K, small arrows) are murine. Stromal cells at the abluminal side of sinusoids, or interspersed among hematopoietic cells, are human (J–L, large arrows), as are bone-lining cells (L, double small arrow). hac, hydroxyapatite carrier.

Secondary Passage of CD146$^+$ CFU-Fs

Having shown that CD146$^+$ cells could give rise to heterotopic stromal cells with anatomy and phenotype identical to those of the originally explanted cells, we sought evidence that CFU-Fs could be secondarily passaged as further indication of the self-renewal capacity of CD146$^+$ stromal cells. Additional transplants were generated with cell strains originating from a limited number of CFU-Fs (10 and 12, respectively, in two experiments). At 8 weeks, transplants were harvested and collagenase-digested to generate single-cell suspensions. Total human cells were magnetically sorted, either after short-term culture (Figure 6A) or directly from the fresh cell suspension (Figure 6B), based on hCD44 expression, which allowed for recovery of $\sim 2 \times 10^4$ and $\sim 1.5 \times 10^4$ human cells, respectively (Table S6). Aliquots of 2×10^3 and 5×10^3 cells were plated at clonal density. One and six discrete colonies were observed and harvested at 2 weeks, which translates into an estimated number of secondary CFU-Fs of 10 and 18, respectively, in the original cell suspensions (Table S6). FACS analysis showed homogeneous, high expression of CD146 in all colonies (Figures 6A and 6B).

In separate experiments (Figures 6C and 6D), transplants generated by five clonal strains, each of which was individually expanded from single CFU-Fs, were similarly collagenase-digested to generate cell suspensions. Greater than 1×10^6 total cells were obtained in 2/5 transplants generated by the progeny of a single CFU-F. A $2–5 \times 10^5$ aliquot from each of the five cell suspensions liberated by collagenase was used for FACS analysis and revealed a frequency of hCD146$^+$ cells of 0.09%–0.4% (Table S6). From the two collagenase-released populations with the largest numbers of cells, the remaining aliquots were magnetically sorted directly for human CD146$^+$ cells, resulting in $\sim 2.1 \times 10^3$ and $\sim 4.0 \times 10^3$ cells, and plated in culture at a density of 1.6 cells/cm^2. This resulted in the generation of 3 and 2 secondary CFU-Fs, which translates into an estimated total number of 4 and 3 assayable CFU-Fs from the original total cell suspension, respectively. No colonies were generated from CD146$^-$ cells, plated at the same or higher (160 cells/cm^2) density. All of the colonies generated by the secondary CFU-Fs expressed CD146 at high levels (Figures 6C and 6D). Thus, heterotopic ossicles generated either by multiple CFU-Fs or by single CFU-Fs contained a number of assayable CD146$^+$ CFU-Fs similar to or greater than the number of originally explanted, culture-initiating CD146$^+$ CFU-Fs.

Regulated Production of Ang-1 by CD146$^+$ Stromal Cells

Ang-1 (a ligand of the Tie-2 tyrosine kinase receptor specifically expressed in ECs and HSCs [Davis et al., 1996; Dumont et al., 1992; Hsu et al., 2000; Iwama et al., 1993; Sato et al., 1993]) is produced by MCs in development

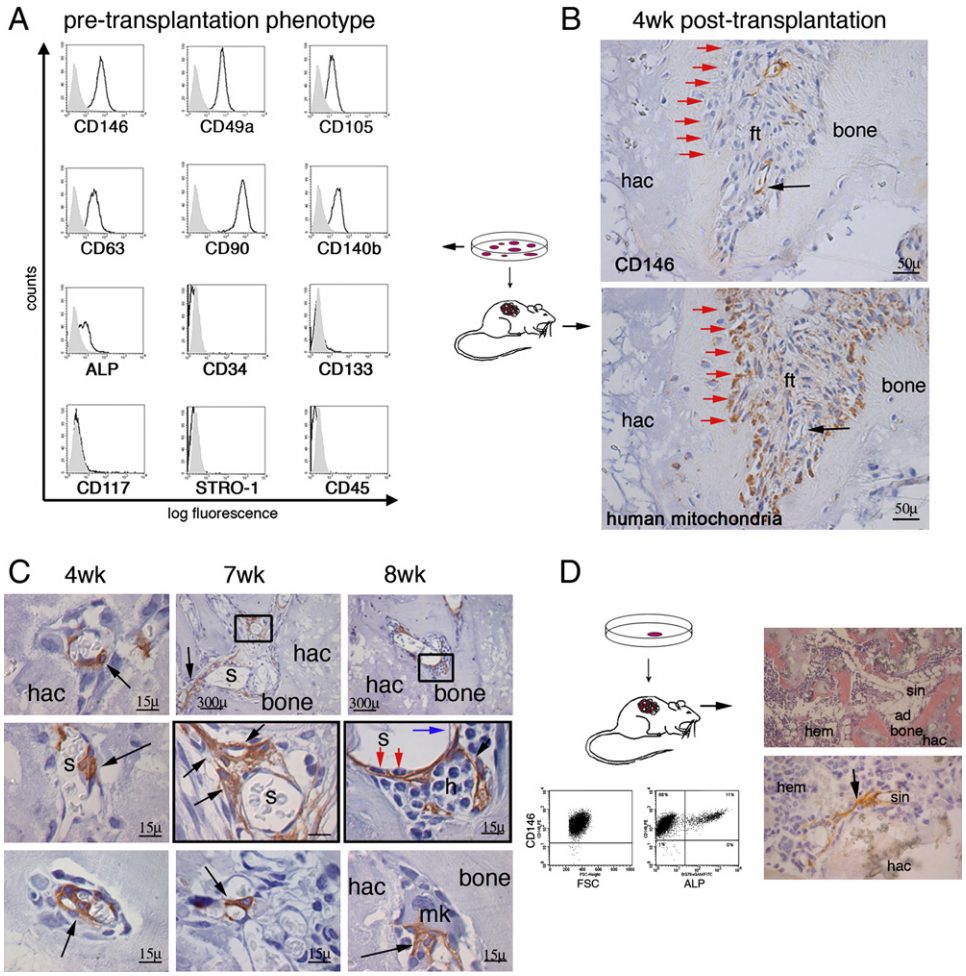

Figure 5. Self-Renewal of CD146$^+$ Cells In Vivo

(A) FACS analysis prior to transplantation. Homogeneous expression of high levels of CD146.

(B) Serial sections of transplants of the same strain at 4 weeks, stained for CD146 and human mitochondria, respectively. The vast majority of human cells in the ossicle, including osteoblasts (red arrows) are CD146$^-$. CD146$^+$ cells are restricted to an adventitial layer in vessel walls.

(C) Development of CD146$^+$ ARCs in the heterotopic BM. At 4 weeks, small mononuclear CD146$^+$ cells associate with blood vessels. At 7 weeks, elongated CD146$^+$ cells are found over and around sinusoids. At 8 weeks, reticular processes of CD146$^+$ cells establish contacts with hematopoietic cells (h). mk, megakaryocyte; blue arrow, endothelium; red arrows, hematopoietic cells between endothelial and CD146$^+$ cells.

(D) Transplantation of a single CFU-F derived cell strain. At 14 days, the colony formed by a single CFU-F was isolated with a cloning cylinder and individually expanded. Bottom left, FACS analysis of the expanded clone at passage 3, demonstrating the homogeneous expression of CD146 (and the coexpression of ALP in a subset of the clonal population). Top right, histology of the heterotopic ossicle (8 weeks) formed by the expanded clonal population. Bone, sinusoids (sin), adipocytes (ad), and hematopoiesis (hem) are shown. Bottom right, human CD146$^+$ adventitial reticular cells in the same heterotopic ossicle.

and regulates microvessel assembly and remodeling in mice (Suri et al., 1996). Reported to be produced by osteoblasts, Ang-1 has also been implicated as a key component of the HSC niche in postnatal murine bone (Arai et al., 2004). Since our data implicated CD146$^+$ cells in establishment of both the sinusoidal wall structure and the HME in vivo, we investigated Ang-1 expression. In human BM in situ, Ang-1 immunoreactivity was restricted to ARCs with no signal over bone surfaces where differentiated osteoblasts reside (Figure 7A). In the heterotopic ossicles prior to the establishment of hematopoiesis, Ang-1 was localized to mesenchymal cells around sinusoids but not to osteoblasts (Figure 7B). Once hematopoiesis was established, ARCs adjacent to sinusoids expressed Ang-1, whereas Tie-2 was localized to sinusoidal endothelium and to rare, small cells of hematopoietic habit, adjacent to the sinusoidal wall (Figure 7C). Undifferentiated CD146$^+$ BMSCs produced abundant Ang-1 in vitro but did not express Tie-2. When BMSCs were induced to differentiate into osteoblasts in vitro, both Ang-1 and CD146 expression were potently downregulated (Figures 7D–7G), in agreement with our in vivo data. Interestingly, gene knockdown of either CD146 or Ang-1 in CD146$^+$ stromal cells significantly interfered with their ability to direct the

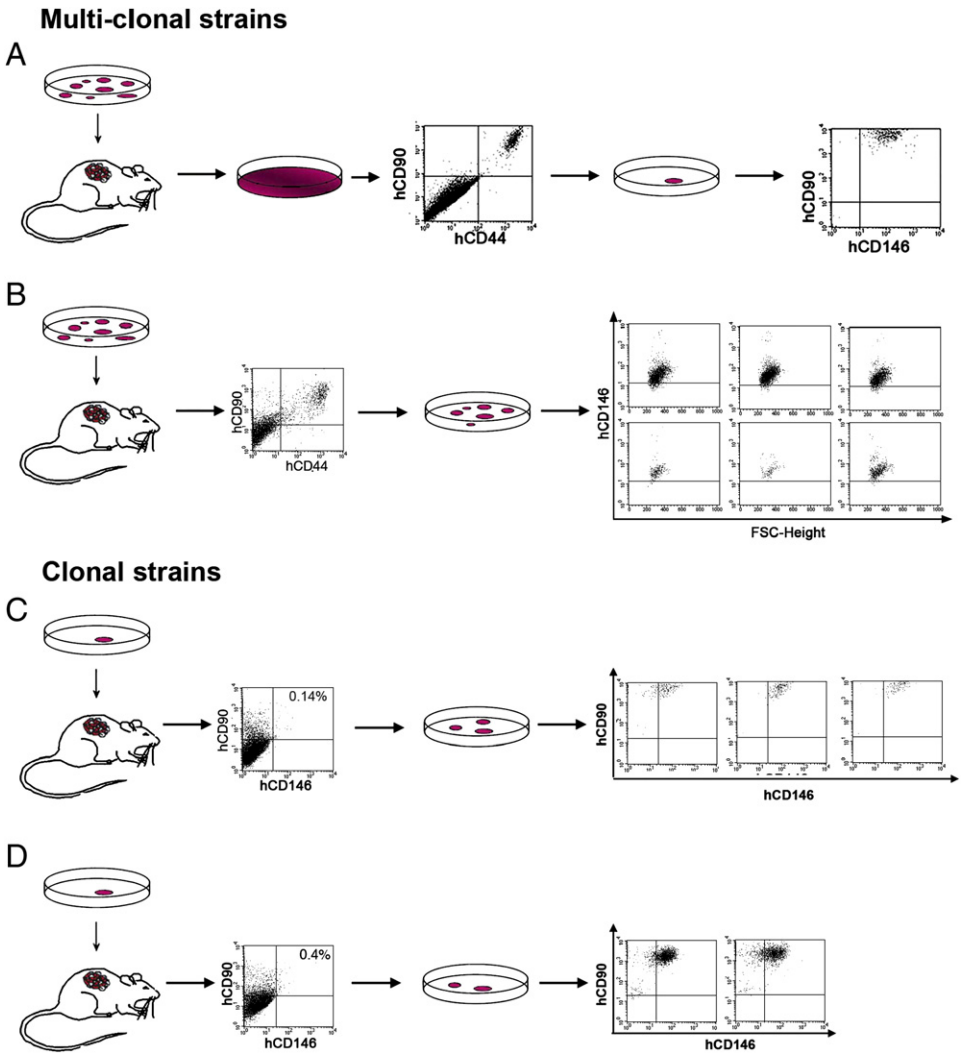

Figure 6. Secondary Passage of CD146$^+$ CFU-Fs

CD146$^+$ CFU-Fs were recovered from heterotopic ossicles generated by transplanting the progeny of either a limited number of CFU-Fs (A, n = 10; B, n = 12) or a single CFU-F (C and D). In (A) and (B), hCD90$^+$/hCD44$^+$ cells were sorted after short-term culture (A) or directly from the collagenase-generated cell suspension (B). All colonies obtained by plating sorted cells at clonal density were homogeneously CD146^{high}. In (C) and (D), CD146$^+$ cells were sorted directly from the cell suspension obtained from single CFU-F-generated ossicles. All colonies (C, 3 colonies; D, 2 colonies) obtained by replating the sorted cells at clonal density expressed CD146 at high levels. No colonies were obtained from the CD146$^-$ population (not shown).

assembly and remodeling of pseudovascular structures in vitro (Figure S7), suggesting that both CD146 and Ang-1 expressed in stromal cells may participate in stromal-endothelial interactions. Both CD146 and Ang-1 were also downregulated by FGF-2 (Figure S8) in the context of the complex effects exerted by this factor on the growth and differentiation of CD146$^+$ cells in culture. Notably, in transplants generated with FGF-2-treated CD146$^+$ cells, the ability of the latter to establish CD146$^+$ stromal cells and the HME in vivo was abrogated, while their ability to form bone remained unscathed (Figure S8), indicating that the two abilities can be dissociated from one another in a single-cell population otherwise competent to both functions.

DISCUSSION

The notion that BM includes skeletal progenitor (stem) cells and the notion that BM stroma provides cues for homing, maintenance, proliferation, and maturation of hematopoietic progenitors both emanate from the same classical transplantation experiments. The multipotency of at least a subset of CFU-Fs supports the view that a second type of stem cell (skeletal [Bianco and Robey, 2004], stromal [Owen and Friedenstein, 1988], or "mesenchymal" [Caplan, 1991]) coexists with HSCs in BM. Due to the lack of markers suited to bridge the gap between in situ, ex vivo, and in vivo observations, the in situ counterpart of CFU-Fs has previously remained unknown, and the

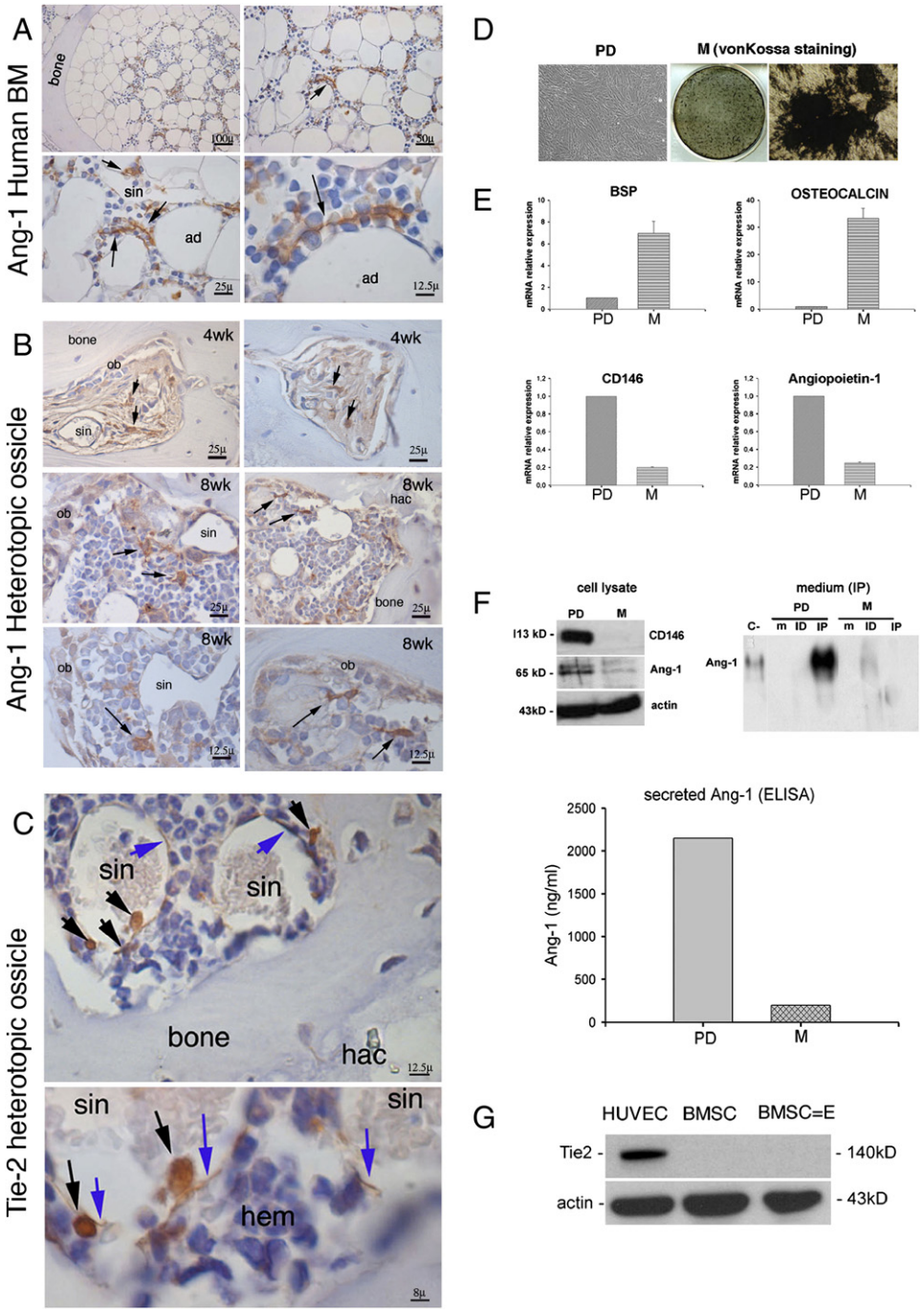

Figure 7. Expression of Ang-1 and Tie-2

(A) Ang-1 immunoreactivity in sections of human BM is restricted to ARCs (arrows) and absent over bone surfaces. ad, adipocytes.

(B) Localization of Ang-1 to ARCs in heterotopic ossicles. Prior to establishment of hematopoiesis (4 weeks post-transplantation), Ang-1 immunoreactive-mesenchymal cells reside in presumptive marrow spaces in the vicinity of developing sinusoids. After establishment of hematopoiesis, reticular cells in the hematopoietic space and in the vicinity of sinusoids express Ang-1. Osteoblasts are not labeled. ob, osteoblasts; hac, hydroxyapatite carrier; sin, sinusoid; arrows, ARCs.

(C) Expression of Tie-2 in heterotopic ossicles is restricted to sinusoidal endothelium (blue arrows) and to rare small mononuclear cells adjacent to the luminal or abluminal side of endothelium (black arrows).

(D and E) Expression and regulation of CD146 and Ang-1 in $CD146^{+}$ cells induced to differentiate into osteoblasts in vitro. PD, pre-differentiation; M, mineralization conditions. (D) Matrix mineralization. (E) Upregulation of markers of mature osteoblasts (BSP, osteocalcin) and downregulation of CD146 and Ang-1 mRNAs (qPCR, fold change over PD). Error bars indicate ± standard deviation (SD).

very self-renewal of stromal progenitors, a defining characteristic of stem cells, has not been formally demonstrated. Expression of high levels of CD146, a cell adhesion molecule of the immunoglobulin superfamily expressed in a restricted range of normal cells (Shih, 1999), identifies all ex vivo assayable CFU-Fs, and a specific subset of stromal cells in situ. Explantable CFU-Fs exhibit the same phenotype as adventitial reticular cells (ARCs), which reside in bone marrow sinusoids next to the endothelial layer, strongly indicating that ARCs are in fact the cells explanted ex vivo as CFU-Fs. We have now shown that following transplantation of $CD146^+$ stromal cells, a small subset retain CD146 expression, dynamically associate with developing sinusoids, and eventually regenerate heterotopic human cells with the anatomy and phenotype of ARCs.

We have also shown that transplantation of cell populations derived from either a limited number of $CD146^+$ CFU-Fs or single $CD146^+$ CFU-Fs results in the re-establishment, in the heterotopic ossicles, of $CD146^+$ CFU-Fs that can be secondarily passaged and directly assayed. By providing evidence for the ability of $CD146^+$ stromal cells to function as self-renewing, clonogenic skeletal progenitors, our data outline the long sought anatomical identity of SSCs ("mesenchymal" stem cells) in human BM and a crucial feature of their phenotype. While our data provide evidence for the self-renewal and multipotency of $CD146^+$ CFU-Fs, a larger-scale study would be necessary to accurately determine the actual frequency of in vivo assayable, multipotent, and self-renewing clonogenic progenitors within our population of phenotype-defined cells. Even though such frequency would appear to be high based on our data (50%), the relative weight of inherent biological heterogeneity, versus heterogeneity relative to the specific experimental assay and its constraints (including culture and transplantation conditions), remains to be further analyzed.

As portrayed in our system, self-renewal of adventitial reticular cells originally explanted as $CD146^+$ CFU-Fs is inscribed into dynamic organogenic events, which are part of the stepwise reconstitution, in vivo of the HME. The establishment of subendothelial ARCs at heterotopic marrow sinusoids involves the interaction of transplanted cells with host endothelial cells and the remodeling and maturation of sinusoidal vessels. A lead to the identification of mechanisms dictating the establishment of skeletal progenitors in BM during organogenesis, and their regeneration in our model, can be found in this context. In the maturation phase of angiogenesis, the mitotic quiescence of the perivascular mesenchymal cells that are recruited to a subendothelial mural cell fate is induced via direct interaction with endothelial cells and may be mediated by TGF-β1, locally activated at the interface of endothelial and subendothelial cells (Antonelli-Orlidge et al., 1989; Jain, 2003; Sato and Rifkin, 1989). Conceivably, the establishment of quiescent skeletal progenitors at the sinusoidal wall during organogenesis may depend on mechanisms similar to those establishing mitotically quiescent mural cells in other tissues, a view that is consistent with our in vivo and ex vivo data.

How the ability to generate differentiated bone-forming cells and bone tissue relates to the ability to support hematopoiesis (both shared by the BM stroma as a whole) has long remained elusive. Recent data suggest that osteoblasts (differentiated bone-forming cells residing on bone surfaces) directly maintain a niche for HSCs (Calvi et al., 2003; Moore and Lemischka, 2006; Zhang et al., 2003), a view that would easily account for the fact that transplantation of BM stroma leads to the formation of hematopoiesis-accommodating bone. In search for the specific cell type in BM stroma that is capable of establishing the HME at heterotopic sites, however, we have shown that this ability is not synonymous with the ability to generate differentiated osteoblasts and bone tissue in vivo. Cell strains originating from $CD146^{high/bright}$ clonogenic progenitors in BM form bone and transfer the HME in vivo. $CD146^{low/dim}$ cell strains originating from specific anatomical compartments of bone other than BM space (trabecular bone or periosteum) do establish differentiated osteoblasts and bone upon in vivo transplantation but do not transfer the HME. Furthermore, in a cell population competent to form bone and establish the HME in vivo, the two functions can be experimentally dissociated from one another, as seen, for example, as the effect of FGF-2 treatment in our data.

Establishment of the HME at heterotopic sites occurs via a defined developmental sequence in which bone formation regularly precedes the appearance of a heterotopic bone marrow stroma and ultimately of heterotopic hematopoiesis. Whereas the establishment of osteoblasts and bone may be necessary as part of this developmental sequence, additional events precede the establishment of hematopoiesis within bone at heterotopic sites: the remodeling of the local vasculature into a sinusoidal system and the establishment of a $CD146^+$ stromal population at the sinusoidal wall. Although these events involve the interaction of different cell types, including both donor- and host-derived (endothelial) cells in our system, transplanted $CD146^+$ stromal progenitors critically contribute to their unfolding in vivo. Of note, neither a sinusoidal system nor a local population of human $CD146^+$ stromal cells are established in vivo when human $CD146^-$ cell populations are transplanted that are competent to generate bone and osteoblasts in vivo but unable to transfer the HME.

The link between establishment of subendothelial cells in BM and establishment of the HME finds in Ang-1 (Davis et al., 1996) an important molecular correlate. As the

(F) Western analysis demonstrating depletion of CD146 and Ang-1 protein and ELISA demonstrating depletion of secreted Ang-1. m, medium; ID, immunodepleted medium; IP, immunoprecipitated medium.
(G) Tie-2 expression in HUVEC and lack of Tie-2 expression in BMSCs and BMSCs exposed to endothelial differentiation medium (BMSC-E).

ligand of the Tie-2 receptor that is specifically expressed in ECs and HSCs, Ang-1 plays pivotal roles both in angiogenesis and hematopoiesis. Distinct from the growth-promoting effects of VEGF, the role of Ang-1 in angiogenesis is specifically related to establishment of MCs and to remodeling of vascular plexuses (Suri et al., 1996). Considering that in development Ang-1 is both a product and a regulator of pericytes/mural cells, Ang-1 expression in BM CD146$^+$ cells is consistent with their overall "mural" cell phenotype and subendothelial position. We have shown that in human BM and in heterotopic ossicles in vivo, human stromal cells are major producers of Ang-1, and Ang-1 production is regulated in vitro when stromal cells are induced to differentiate into osteoblasts or exposed to angiogenesis-regulating factors, such as FGF-2, that act in vivo on the peri-endothelial mesenchyme. Vascular remodeling is a significant component of BM organogenesis. This process establishes a unique system of large, slow flow sinusoids conducive for bidirectional cell traffic between BM and peripheral blood. As portrayed in our in vivo transplantation system, timed remodeling of capillaries into sinusoids, before the establishment of hematopoiesis, is coupled to the physical association of CD146$^+$ subendothelial cells with nascent or growing vessels. Ang-1 may contribute to sinusoid remodeling in postnatal BM when systemically delivered (Hattori et al., 2001), and we have observed that BMSC-directed patterning and remodeling of pseudovascular structures in vitro is altered by Ang-1 (and CD146) gene silencing. This suggests that Ang-1 (and CD146), expressed locally in BMSCs, may be part of the molecular machinery regulating vascular remodeling through a local interaction of endothelial and subendothelial cells, which may contribute to the organization of the unique vascular structure of the bone marrow.

Current evidence suggests that Ang-1 also directly contributes to HSC regulation (Arai et al., 2004) by interacting with HSC-expressed Tie-2. A body of evidence also suggests that endosteal (Calvi et al., 2003; Zhang et al., 2003) and sinusoidal surfaces (Kiel et al., 2005), but also CXCL12-expressing "reticular" cells within the hematopoietic space (Sugiyama et al., 2006), may represent specific sites of HSC regulation ("niches"). Our data show that CD146$^+$ stromal progenitors indeed physically coincide with reticular cells and express CXCL12, Ang-1, and multiple other gene products that have been implicated in HSC regulation. As osteoblast progenitors, CD146$^+$ stromal cells generate osteoblasts, which form bone and are regarded as critical components of an endosteal HSC niche. As (self-renewing) progenitors of sinusoidal adventitial reticular cells, CD146$^+$ stromal cells contribute to the organization, and become an integral part, of the structure of sinusoidal walls, in the vicinities of which HSCs have been directly localized (Kiel et al., 2005). Skeletal progenitors residing over sinusoids may thus contribute to hematopoietic regulation within the BM—either directly at the sinusoidal wall where they reside as adventitial reticular cells or through their osteoblastic progeny at endosteal surfaces. For example, CD146$^+$ subendothelial cells expressing HSC regulators such as Ang-1 or CXCL12 would be strategically positioned to facilitate the homing of blood borne hematopoietic progenitors to the marrow environment or to contribute to a steady-state sinusoidal niche where HSCs can be localized. By generating, or contributing to, functionally distinct cell types (osteoblasts and ARCs) and structures (bone surfaces and sinusoidal abluminal surfaces), CD146$^+$ skeletal progenitors play a pivotal role in the development of the HME, as recapitulated in our in vivo system, and contribute to establishing and organizing the very diversity of physiologically important and spatially distinct microenvironments within the BM. Our data also indicate that properties of CD146$^+$ subendothelial cells, such as Ang-1 expression, that are relevant to hematopoietic regulation may be modulated when skeletal progenitors are themselves recruited to cell proliferation or osteogenic differentiation or exposed to vasculogenic cues. These cellular events are inscribed, in vivo, in fundamental organogenic processes such as skeletal growth, lifelong bone remodeling, and adaptation, to which skeletal progenitors critically contribute. Hence, the complex interplay of osteogenesis and hematopoiesis in development, physiology, and disease may be seen as rooted into a unique functional interplay of two systems of progenitor/stem cells that takes place in the bone marrow environment at specific sites.

EXPERIMENTAL PROCEDURES

Reagents

Antibodies for cell sorting and flow cytometry are listed in Table S1 and for immunohistochemistry and western blotting in Table S7. Primers for RT-PCR are listed in Table S8.

Culture and Characterization of Cell Strains

BMSCs, PCs, HTBs, and stromal cells from the nonhematopoietic bone marrow of fibrous dysplastic bone (FD) were isolated by established methods (Bianco et al., 1998; Kuznetsov et al., 1997; Miura and O'Driscoll, 1998; Robey and Termine, 1985). Samples were obtained with informed consent per institutionally approved protocols. Human foreskin fibroblasts (SFs) were from A. Orecchia, IDI, Rome, Italy. Human muscle fibroblasts (MFs) were from G. Cossu, DIBIT-HSR, Milan, Italy. After primary culture, all strains were cultured under identical conditions in α-MEM (Invitrogen)/20% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, prior to analysis. Expression of markers was assessed using a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson Biosciences, San Diego, CA). In vitro differentiation assays were done by established methods (Bianco et al., 2006).

Cell Sorting

1×10^7 freshly isolated BMNCs were resuspended in HBSS/30 mM HEPES (Sigma, St. Louis, MO), 100 U/ml penicillin, 100 μg/ml streptomycin, 1% BSA (Sigma) and incubated on ice for 30 min. Cells were pelleted in HBSS/2 mM EDTA, 1% BSA, resuspended in 1 ml blocking buffer, and incubated with anti-CD45 conjugated magnetic beads (Miltenyi Biotec, Auburn, CA) for 20 min on ice. Cells were separated into CD45$^-$ and CD45$^+$ fractions using a MiniMACS magnetic column separation unit per the manufacturer's instructions (Miltenyi). CD45$^-$ cells were incubated with PE-conjugated anti-CD146 antibody, and CD146$^+$ and CD146$^-$ cells were separated using a FACS DIVAntageSE flow cytometer (BD Biosciences Labware, San Diego, CA).

CFE Assays and CFU-F Cultures

CFE was assessed as described (Kuznetsov et al., 1997). Plating densities were 0.1–10 × 10^3/cm^2 for total BMNCs and 1.6–1.6 × 10^4 cells/cm^2 for separated fractions of BM cells. Colonies (>50 cells) were counted after 14 days. Multiclonal strains were established by passaging all colonies obtained in primary CFU-F cultures. Individual colonies (clones) were isolated from primary CFU-F cultures using cloning cylinders (Kuznetsov et al., 1997). Nonclonal BMSC cultures were obtained by passaging primary cultures established at nonclonal density (>10^5 total nucleated cells/cm^2).

Histology

Heterotopic ossicles were processed as reported (Bianco et al., 1998). Sections of human iliac crest biopsies (three subjects) with normal BM were cut from archival paraffin blocks on file in our department. Human specificity of the CD146 antibody was verified on sections of mouse bone/BM and of heterotopic ossicles formed by murine BMSCs (Kuznetsov et al., 2004). Immunolocalization was performed using standard immunoperoxidase (DAB reaction) and sections were counterstained with hematoxylin.

In Vivo Transplantation

In vivo transplantation of different cell strains was performed as reported (Krebsbach et al., 1997; Kuznetsov et al., 1997). All animal procedures were approved by the relevant institutional committee. 2 × 10^6 cells were allowed to attach to hydroxyapatite/tricalcium phosphate particles (40 mg, 100–200 μm; Zimmer, Warsaw IN) and embedded in a fibrin gel. Carrier-cell constructs, and carrier alone as control, were transplanted subcutaneously into 8- to 15-week-old female nih/nu/xid/bg mice (Harlan-Sprague Dawley, Inc., Indianapolis, IN).

Secondary Passage of CD146$^+$ CFU-Fs

Cell cultures were initiated either from a limited number of CFU-Fs (10 and 12 in two experiments) or from single CFU-Fs (n = 5). Heterotopic ossicles were harvested at 8 weeks, washed in HBSS/30 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and digested twice with 100 U/ml *Chlostridium histolyticum* type II collagenase (Invitrogen) in PBS/3 mM $CaCl_2$ for 40 min at 37°C. 5 × 10^5 cells obtained from the two digestions were used for FACS analysis of hCD146 expression. Cell suspensions derived from multiclonal generated ossicles were used to magnetically separate human cells based on hCD44 expression using MiniMacs (Miltenyi), either after short-term culture or directly from the fresh cell suspension. hCD44$^+$ cells (~20,000 and ~15,000 in two experiments) were recovered, resuspended in medium, and 2,000 and 5,000 cells, respectively, were plated in culture at clonal density (1.6 cells/cm^2). Cultures were scored for colony formation at 2 weeks. The discrete colonies obtained were harvested and analyzed by FACS for expression of hCD90, hCD44, and hCD146. Cell suspensions obtained by collagenase digestion of ossicles generated by transplanting the progeny of single CFU-Fs were used to sort hCD146$^+$ cells directly. These were then replated at clonal density (1.6 cells/cm^2) to assay for secondary CFU-Fs. The discrete colonies obtained were harvested and analyzed for expression of hCD90, hCD44, and hCD146.

Supplemental Data

Supplemental Data include Supplemental Results, Supplemental Experimental Procedures, eight figures, and nine tables and can be found with this article online at http://www.cell.com/cgi/content/full/131/2/324/DC1/.

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