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Human umbilical cord blood-borne fibroblasts contain marrow niche precursors that form a bone/marrow organoid *in vivo*

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ABSTRACT

Human umbilical cord blood (CB) has attracted much attention as a reservoir for functional hematopoietic stem and progenitor cells, and, recently, as a source of blood-borne fibroblasts (CB-BFs). Previously, we demonstrated that bone marrow stromal cell (BMSC) and CB-BF pellet cultures make cartilage *in vitro*. Furthermore, upon *in vivo* transplantation, BMSC pellets remodelled into miniature bone/marrow organoids. Using this *in vivo* model, we asked whether CB-BF populations that express characteristics of the hematopoietic stem cell (HSC) niche contain precursors that reform the niche. CB ossicles were regularly observed upon transplantation. Compared with BM ossicles, CB ossicles showed a predominance of red marrow over yellow marrow, as demonstrated by histomorphological analyses and the number of hematopoietic cells isolated within ossicles. Marrow cavities from CB and BM ossicles included donor-derived CD146-expressing osteoprogenitors and host-derived mature hematopoietic cells, clonogenic lineage-committed progenitors and HSCs. Furthermore, human CD34$^+$ cells transplanted into ossicle-bearing mice engrafted and maintained human HSCs in the niche. Our data indicate that CB-BFs are able to recapitulate the conditions by which the bone marrow microenvironment is formed and establish complete HSC niches, which are functionally supportive of hematopoietic tissue.

KEY WORDS: Umbilical cord blood, Cord blood-borne fibroblasts, Bone marrow organoid, Hematopoietic stem cell niche

INTRODUCTION

The current view is that multipotent skeletal stem cells, a subset of bone marrow stromal cells (BMSCs) within the bone marrow (BM) compartment, are the prime candidate for establishing, transferring and maintaining the hematopoietic stem cell (HSC) niche, a unique niche made of two stem cells (Sacchetti et al., 2007; Méndez-Ferrer et al., 2010; Bianco, 2011). Whether other stromal cells or blood-borne fibroblasts derived from different adult or fetal sources share this property with BMSCs and are similarly able to recreate a completely humanized hematopoietic niche is not known. Using an experimental model in which cells are resuspended in a Matrigel equivalent, Reinisch et al. compared the capacity of cells derived from different sources (bone marrow stroma, white adipose tissue, umbilical cord, skin) to give rise to a functional hematopoietic niche and concluded that BMSCs, exclusively, have the ability to form a heterotopic bone/marrow organoid (Reinisch et al., 2015).

Umbilical cord blood (CB) is an intriguing source of hematopoietic and non-hematopoietic progenitors. Within the non-hematopoietic fraction, a rare population of cells that can become adherent, defined as cord blood-borne fibroblasts (CB-BFs), can be isolated. The question regarding their origin is still unanswered, as it is not completely understood whether these cells normally circulate in cord blood or whether they are skeletal progenitors located at sites of active skeletal growth that accidentally spill over into the bloodstream.

CB-BFs exhibit a morphological, phenotypical and molecular profile that is distinct from other cells of fetal and adult origin (Secco et al., 2009; Bosch et al., 2013; Pievani et al., 2014; Kern et al., 2006; Bieback et al., 2008; Rebelatto et al., 2008). *In vitro*, CB-BFs display osteogenic and chondrogenic differentiation potential (Pievani et al., 2014), whereas they have a reduced or virtually absent capacity to generate adipocyte-like cells (Kern et al., 2006; Goodwin et al., 2001; Zhang et al., 2011; Bieback et al., 2008; Karagianni et al., 2013). When transplanted *in vivo* with osteoconductive ceramic scaffolds, CB-BFs are able to form histology-proven bone (Zhang et al., 2011; Liedtke et al., 2016; Sacchetti et al., 2016). Interestingly, we have also demonstrated that CB-BFs consistently form cartilage in open heterotopic transplants, a property that is usually not observed in BMSCs. However, unlike BMSCs, which have the specific capacity of generating bone and a bone marrow stroma that supports functional hematopoiesis, CB-BFs do not establish a hematopoietic microenvironment in ceramic scaffold-based transplants (Sacchetti et al., 2016).

Recently, we have developed an *in vivo* experimental model in which the ability of human BMSCs to generate a bone/bone marrow organ was optimized in the complete absence of any exogenous synthetic scaffold/carrier/biomaterial. In this system, cartilage pellets, produced *ex vivo* and subsequently transplanted *in vivo*, remodelled into miniature ossicles with perfect architecture (Serafini et al., 2014). These pellet-derived ossicles include donor-derived bone and marrow stroma, as a result of phenotypical reversion of differentiated chondrocytes, that become subsequently filled by a functional hematopoietic tissue provided by the host.

In this study, we transplanted chondroid rudiments generated *in vitro* from CB-BFs to explore the ability of this cell population

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to establish a vascularized, extramedullary hematopoietic microenvironment *in vivo*. Moreover, we aimed to compare ossicles (i.e. rudiments including bone and BM) obtained from CB-BFs and BMSCs (hereafter termed CB ossicles and BM ossicles, respectively) with respect to their architectural and cellular features, and their ability to support both murine and human HSC homing and maintenance. Finally, we integrated our *in vivo* findings with *in vitro* data aimed at evaluating the expression of HSC maintenance genes and the capacity of CB-BFs to support human HSCs.

RESULTS

CB-BFs are skeletogenic and form pellet-derived ossicles with peculiar histological features

We evaluated the skeletogenic potential of BMSCs and CB-BFs by performing quantitative gene expression of known osteogenic and chondrogenic genes at baseline in monolayer cultures without addition of any inducing factors. We found a similar expression of *RUNX2*, *SPP1*, *BGLAP*, *ALPL*, *ACAN*, *RUNX3* and *DLX6* in both BMSCs and CB-BFs (Fig. 1A). We further verified the skeletogenic property of CB-BFs *in vivo*, evaluating their capacity to generate pellet-derived heterotopic ossicles, in comparison with control BMSCs. For this purpose, we established pellet cultures from CB-BFs and BMSCs, and incubated them with TGFβ1 for 3 weeks prior to *in vivo* transplantation into the subcutaneous tissue of immunodeficient mice. Untransplanted cartilage pellets obtained at the end of the culture period from both CB-BFs and BMSCs showed a similar histological appearance (Fig. 1C). Both types of organoids consisted of unmineralized cartilagenous tissue (negative von Kossa staining, data not shown) in which similar levels of transcripts for proteoglycans and chondrocyte-associated markers, such as *COL2A1*, *COL10A1*, *SOX9* and *ACAN* were detected (Fig. 1B). However, the mean area of the untransplanted cartilage pellets obtained from CB-BFs was significantly greater than that of the pellets generated from BMSCs (2.42±0.81 mm^2 versus 0.84±0.46 mm^2; P=0.0066) (Fig. 1C, right).

Histological analysis of the transplants harvested after 8 weeks *in vivo* demonstrated that complete heterotopic ossicles (i.e. structures including bone and BM) had similarly developed in samples generated by CB-BFs and by BMSCs (n=44 and 99, respectively). The ossicles obtained from CB-BFs mimicked those obtained from BMSCs (Fig. 1D). They demonstrated a striking architectural resemblance to a normal bone/marrow organ and consisted of an outer shell of cortical bone encasing spaces filled with hematopoietic tissue, including erythroid, myeloid and megakaryocytic lineages. However, quantitative histological analysis revealed some differences between the two types of ossicles. As with untransplanted cartilage pellets, the ossicles generated by CB-BFs were significantly larger in size compared with those generated by BMSCs [3.09±0.88 mm^2 (mean±s.d.) versus 0.85±0.26 mm^2; P=0.04] (Fig. 1D, right). Interestingly, this dimensional discrepancy occurred even though the proportion of cells in S phase in cultures (at passages 3-4) and positivity for the cell proliferation antigen, Ki-67, in cartilage pellets (after 1 week of differentiation) were indistinguishable between CB-BFs and BMSCs (cells in S phase: CB-BFs versus BMSCs, 16.19±2.27% versus 19.00±2.93%; P=0.21; percentage of Ki-67 positive cells: CB-pellet versus BM-pellet, 7.48±4.90% versus 6.74±4.73%; P=0.79) (data not shown).

In addition, compared with the ossicles generated by BMSCs, the fraction of BM occupied by adipocytes was significantly lower in the ossicles generated by CB-BFs (CB-BFs versus BMSCs, 1.44±0.68 versus 12.82±7.28; P=0.04) (Fig. 1E). No significant difference was detected by comparing the size of the cartilage pellets with the size of the corresponding ossicles generated by either CB-BFs or BMSCs (data not shown). In other words, the size of cartilage pellets did not change after *in vivo* transplantation.

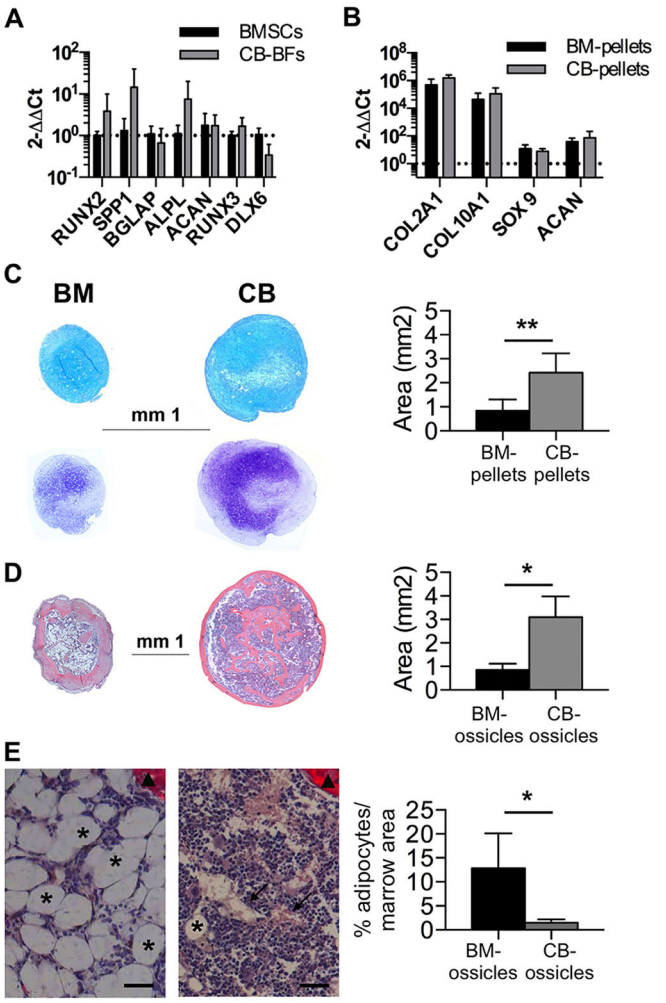

Fig. 1. Chondroid pellets obtained from CB-BFs can generate heterotopic ossicles upon *in vivo* transplantation. (A) Baseline expression of osteogenic- and chondrogenic-specific genes in BMSCs and CB-BFs (n=3). (B) Upregulation of cartilage-related genes detected by quantitative RT-PCR after 21 days of chondrogenic induction in BMSC (n=3) versus CB-BF pellets (n=6). (C,D) Representative histological images of untransplanted cartilage pellets and ossicles obtained from BMSCs (left) and CB-BFs (right) are shown (C, upper, Alcian Blue; C, lower, Toluidine Blue; D, Haematoxylin and Eosin). Graphs show histomorphometric analysis of the areas of both the untransplanted cartilage pellets (C, right) and of the ossicles (D, right) obtained from BMSCs compared with CB-BFs (n=6 versus n=3 pellets, respectively; n=5 versus n=3 ossicles, respectively). Scale bars in C,D: 1 mm. (E) Representative histological images of the BM tissue in ossicles generated by transplantation of a cartilage pellet from either BMSCs (left) or CB-BFs (middle) are shown (Sirius red stain). Graph shows the fraction of BM occupied by adipocytes in the ossicles generated by BMSCs and CB-BFs (n=5 versus n=3, respectively). The black triangles in both the histological images indicate bone tissue. The arrows in the histological image of CB ossicles indicate sinusoids, which are hard to see in the histological image of BM ossicle due to their compression by the greater amount of adipose tissue (asterisks). Scale bars: 100 µm. P values indicated have been calculated with an unpaired two-tailed t-test (*P<0.05, **P<0.01).

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Development of a complete hematopoietic stem cell niche from CB-BFs

Similar to BMSCs, CB-BFs-derived ossicles demonstrated a striking architectural resemblance to natural bone/marrow organs; i.e. they featured a bone cortex encasing a marrow cavity containing hematopoietic tissue, including systems of marrow sinusoids (Fig. 1E). Bone cells (osteocytes and osteoblasts) were human in origin throughout, as demonstrated by their nuclear immunoreactivity for human-specific Lamin A/C (Fig. 2, top panels); hematopoietic and bone marrow sinusoidal cells (endothelial and peri-vascular cells) did not stain, confirming their murine origin. Furthermore, in CB ossicles, as previously demonstrated for BM ossicles (Serafini et al., 2014), we detected the presence of human CD146-positive stromal cells associated with the vessel wall (Fig. 2, bottom panels). The components of the hematopoietic tissue within the ossicles were analysed after collagenase digestion of CB and BM ossicles. Consistent with the results of the histological analysis, the number of hematopoietic cells harvested per ossicle was significantly higher in CB than in BM ossicles ($5.14\pm0.99\times10^5$ versus $2.80\pm1.14\times10^5$, respectively; P=0.0057) (Fig. 3A). The composition of the collected cells was further analysed by flow cytometry for different hematopoietic lineages, showing similar proportions of erythroid cells (Ter-119$^+$), myeloid cells (Mac-1$^+$, Gr-1$^+$ or Gr1$^{-/low}$) and megakaryocytes (CD41$^+$) in CB and BM ossicles, also in comparison with orthotopic BM of the same animals (P values not significant) (Fig. 3B). Both CB and BM ossicles contained Lin$^-$/Sca-1$^+$/c-Kit$^+$ (LSK) progenitors with a similar frequency (0.134±0.041% versus 0.091±0.021%; P=0.079), which is reduced in comparison with normal murine BM (0.263±0.083%; P<0.05) (Fig. 3C). We also calculated the absolute number of LSK progenitors collected per ossicle, showing that it was significantly higher in CB than in BM ossicles (680.25±216.42 versus 325.80±85.19; P=0.011) (Fig. 3D). Furthermore, to identify and enumerate the hematopoietic progenitor cells, methylcellulose cultures were used. As shown in Fig. 3E, both CB and BM ossicles contained a distribution of progenitor cells comparable with control BM, showing the presence of CFU-GEMM, BFU-E, CFU-GM, CFU-G and CFU-M. Although not statistically significant, there was a trend for an increase in the proportion of progenitor cells derived from CB ossicles compared with BM ossicles. More hematopoietic progenitor cells were present in the ossicles when compared with peripheral blood (PB) harvested from the ossicle-bearing mice, in which we mainly detected BFU-E. Furthermore, a panel of specific markers was used to investigate the presence of HSCs and progenitors in the ossicles (Fig. 3F). Interestingly, in CB and BM ossicles, the frequency of putative long-term HSCs (LSK/CD34$^-$/Flk2$^-$), short-term HSCs (LSK/CD34$^+$/Flk2$^-$) and multipotent progenitors (MMP, LSK/CD34$^+$/Flk2$^+$) displayed a comparable distribution with that found in BM derived from femurs. Similarly, no major differences were observed in megakaryocyte-erythroid [MEP, Lin$^-$/c-Kit$^+$/Sca-1$^-$(LK)/FcγR$^-$/CD34$^-$], common myeloid (CMP, LK/FcγR$^-$/CD34$^+$) and granulocyte-macrophage (GMP, LK/FcγR$^+$/CD34$^+$) progenitors.

Engraftment of human hematopoietic stem cells into CB ossicles

To investigate whether the *in vivo* generated CB ossicles could also support human hematopoiesis, we developed a transplantation protocol, as illustrated in Fig. 4A. Briefly, 3 weeks after transplantation of the pellets (when vascular invasion is initiated and formation of a primitive stroma is emerging), mice were sublethally irradiated and inoculated with human cord blood CD34$^+$-enriched cells (h-CD34$^+$ cells). Six weeks later, ossicles were harvested and analysed for human cell engraftment together with various hematopoietic organs, including BM, spleen and PB of the same animals. Similar percentages of human CD45$^+$ cells were detected by flow cytometry in hematopoietic cells collected from CB and BM ossicles, and comparable with the engraftment observed in BM (Fig. 4B). Human CD45$^+$ cells were also detectable, although at lower levels, in the spleen and PB of engrafted mice (Fig. 4B).

In addition, we evaluated the ability of human hematopoietic progenitor cells to differentiate into committed hematopoietic cells

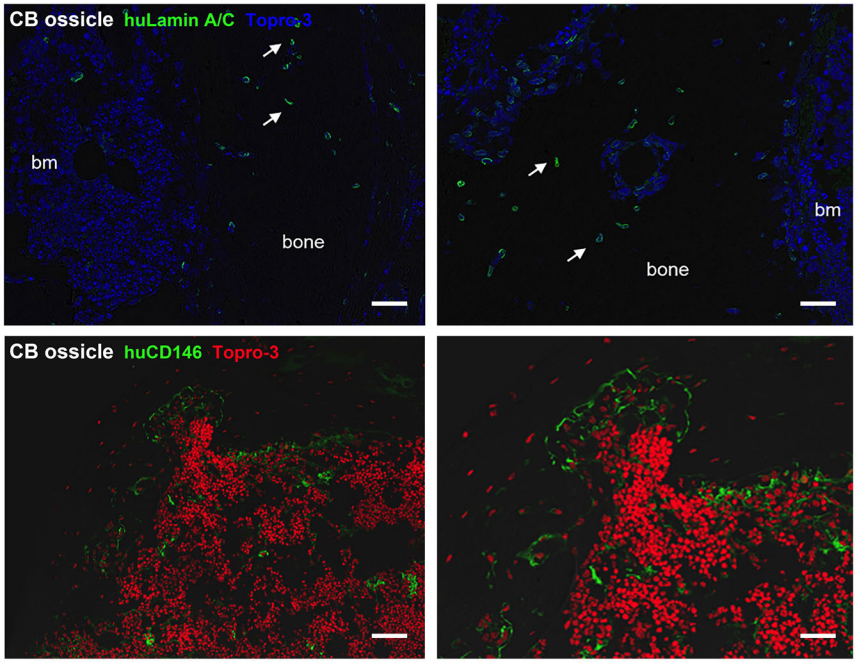

Fig. 2. Features of the stromal compartment in CB-BF-derived ossicles. Confocal fluorescence images to demonstrate the contribution of human CB-BFs cells to the generation of the different tissues within the heterotopic ossicles. Top panels show the expression of huLamin A/C in the nuclear membrane of osteocytes (arrows). Scattered human nuclei are also immunolabelled among the hematopoietic cells, which are negative and, thus, of mouse origin. Bottom panels show the expression of hCD146 in stromal cells associated with the vessel wall within the hematopoietic marrow. Scale bars: 150 µm (top); 100 µm and 50 µm (bottom left and bottom right, respectively). Cell nuclei were stained with Topro-3 iodide.

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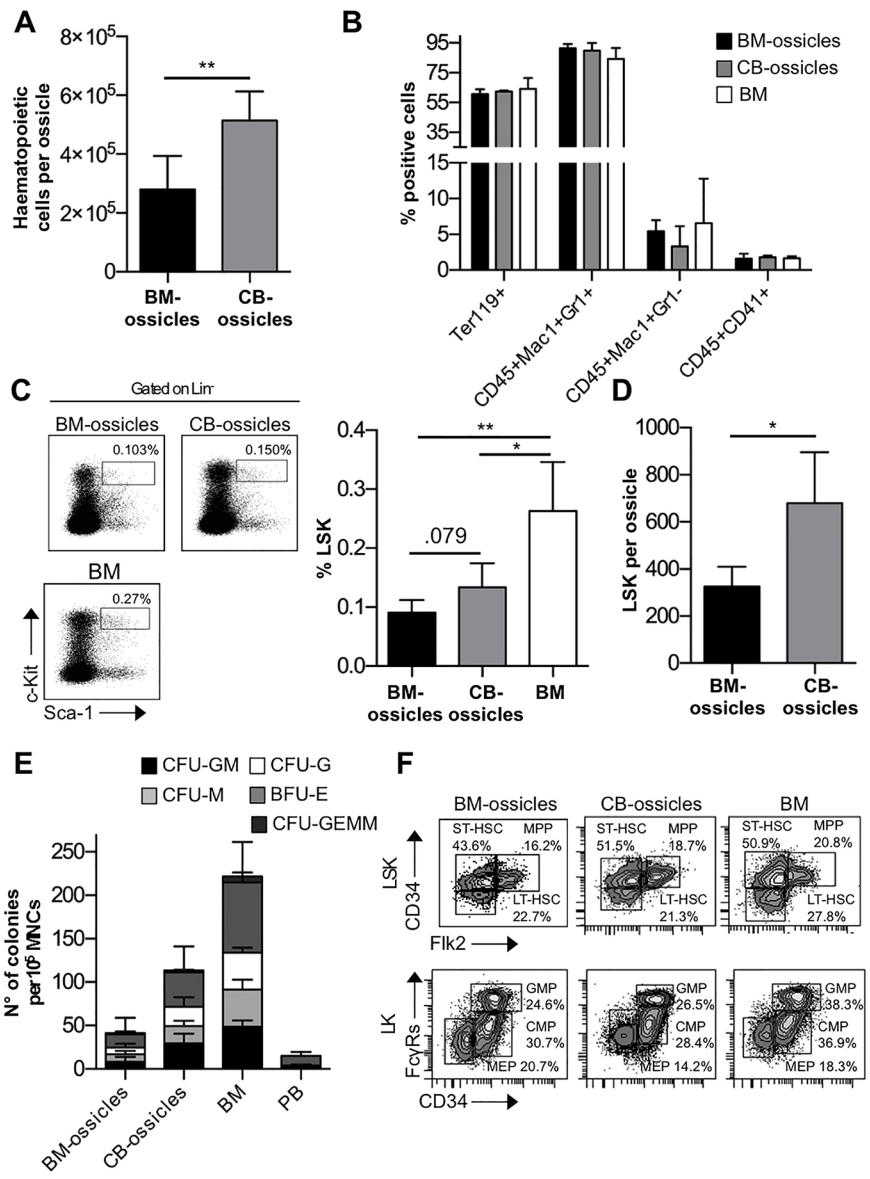

Fig. 3. Features of murine hematopoietic engraftment in heterotopic ossicles. (A) Number of hematopoietic cells harvested from BM ossicles or CB ossicles (n=94 versus 41 ossicles, respectively, from six and five independent experiments, respectively; $^{**}P$<0.01, unpaired two-tailed t-test). (B) Comparison of murine multilineage hematopoiesis in heterotopic BM ossicles and CB ossicles, and in mouse femur BM (n=16 versus 31 ossicles from three independent experiments). (C) Representative profile of LSK cells in ossicles derived from BMSCs and CB-BFs, and mouse femur BM (left). Graph shows frequency of LSK cells from explanted BM ossicles and CB ossicles, and mouse femur BM [n=90 BM ossicles versus 31 CB ossicles from five and four independent experiments; $^{*}P$<0.05, $^{**}P$<0.01, unpaired two-tailed t-test]. (D) Number of LSK cells per ossicle evaluated in explanted cells from BM ossicles and CB ossicles ($^{*}P$<0.05, unpaired two-tailed t-test). (E) Frequency of various colony types generated from hematopoietic cells from BM ossicles, CB ossicles, mouse femur BM and peripheral blood (PB) (n=3 independent experiments). (F) Representative cytofluorometric analysis of murine HSCs and progenitors in mouse femur BM, BM ossicles or CB ossicles. LT-HSCs (long-term HSCs; LSK/CD34^{-}/Flk2^{-}), ST-HSCs (short-term HSCs;LSK/CD34^{+}/Flk2^{-}), MPPs (multipotent progenitors; LSK/CD34^{+}/Flk2^{+}), CMPs (common myeloid progenitors; LK/FcγR^{-}/CD34^{+}), GMPs (granulocyte/monocyte progenitors; LK/FcγR^{+}/CD34^{+}) and MEPs (megakaryocyte/erythroid progenitors; LK/FcγR^{-}/CD34^{-}) were gated as indicated.

of distinct lineages within the ossicles. B cells were robustly engrafted in both types of ossicles and in mouse BM (Fig. 4C). Small percentages of human myeloid cells (CD33^{+}, CD14^{+}, CD64^{+}) were also detectable at comparable levels in BM ossicles, CB ossicles and mouse BM. Similarly, human HSCs (CD34^{+}) were engrafted in ossicles derived from both sources and in mouse BM.

CB-BFs express markers that hallmark the HSC niche and maintain HSCs *ex vivo*

Because culture-expanded human CB-BFs are capable of establishing the hematopoietic microenvironment when transplanted as cartilage pellets into immunodeficient mice, we then assessed their capacity to maintain HSCs *ex vivo*. First, we evaluated their expression of CD146, a marker of clonogenic human skeletal progenitors able to establish the hematopoietic microenvironment (Sacchetti et al., 2007). Human CB-BFs at passages 3-4 showed a similar level and extent of CD146 expression by FACS analysis when compared with BMSCs at the same passage (Fig. 5A). In addition, the ability of single cells (colony-forming unit-fibroblasts) to generate colonies (colony-forming efficiency) was comparable in both populations at passages 3-4, demonstrating that they have a comparable clonogenic capacity and, consequently, progenitor frequency (Fig. 5B). Furthermore, CB-BFs expressed HSC niche regulatory genes, such as *CXCL12*, *VCAM1*, *ANGPT1*, *KITLG*, *SPP1* and *JAG1*, confirming that the HSC niche commitment of CB-BFs is already primed and regulated at the transcriptional level. All of these genes were expressed to a similar extent in CB-BFs and BMSCs, with the exception of *CXCL12* and *VCAM1* (Fig. 5C).

Finally, we assessed their ability to maintain HSCs and progenitors *ex vivo*. To this end, we co-cultured 10^5 h-CD34^{+} cells on an adherent CB-BF and BMSC confluent cultures. After 7 days, the total number of non-adherent hematopoietic cells was reduced, but was similar in BMCSs and CB-BFs co-cultures (4.4±2.4×10^4 versus 4.8±2.1×10^4; P=0.87) (Fig. 5D). Regarding the differentiation state of h-CD34^{+} after 7 days of co-culture, no significant differences in the percentages of CD34^{+}/CD38^{+} and CD34^{+}/CD38^{-} cells were observed under the different conditions. In particular, the more undifferentiated population (CD34^{+}/CD38^{-}) was similarly maintained by both CB-BFs and BMSCs (14.83±4.49% versus 11.03±3.11%; P=0.29) (Fig. 5E).

We then used an *in vitro* clonogenic colony-forming unit (CFU) assay to test the ability of BMSCs and CB-BFs to preserve the

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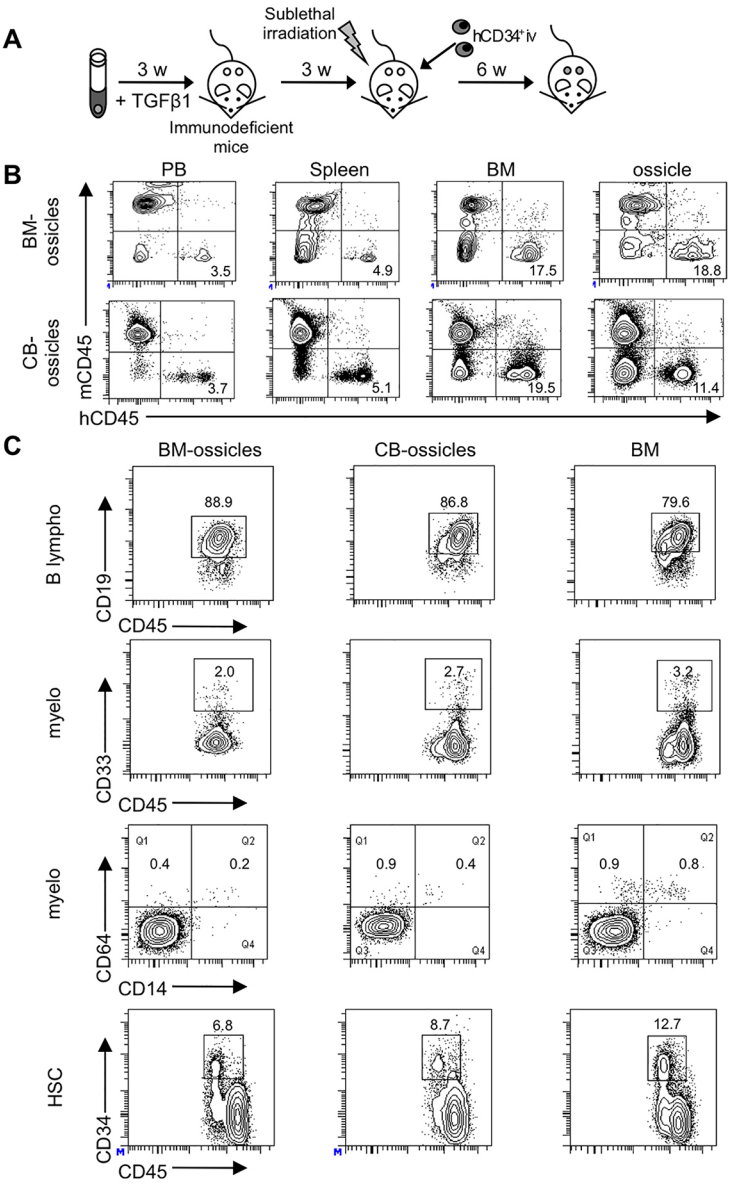

Fig. 4. Features of human hematopoietic engraftment in heterotopic ossicles. (A) Schematic representation of the transplantation protocol. CB-BFs, or BMSCs as control, were induced to form cartilage by culturing as pellets in the presence of TGFβ1 for 3 weeks. Pellets were then transplanted into immunocompromised mice. At 3 weeks after transplantation, mice bearing heterotopic ossicles were sublethally irradiated and transplanted intravenously with human CB-derived CD34$^+$ cells (hCD34$^+$). The engraftment level within mouse femur BM and in the heterotopic ossicles was evaluated 6 weeks later. (B) Representative evaluation of human engraftment in PB, spleen, mouse femur BM and ossicles (derived from BMSCs, *n*=9 and from CB-BFs, *n*=22). (C) Representative cytofluorometric analysis of human multilineage hematopoietic cells harvested from humanized BM ossicles and CB ossicles, and from mouse femur BM.

hematopoietic progenitor cell function of recovered cells. Equal numbers of h-CD34$^+$ cells that had been previously cultured for 7 days with CB-BFs or BMSCs were plated in methylcellulose and hematopoietic colonies were counted after 14 days. No statistical differences in the number and phenotype of CFUs obtained from h-CD34$^+$ cells previously co-cultured with both BMSCs and CB-BFs were observed (Fig. 5F).

A xenotransplantation assay in NSG mice was used to evaluate the *in vivo* SCID-repopulating function of h-CD34$^+$ cells previously co-cultured with BMSCs or CB-BFs, showing similar levels of engraftment in BM and similar capacity to colonize other hematopoietic tissues. In addition, the engraftment composition was comparable between h-CD34$^+$ cells that had been co-cultured with either BMSCs or CB-BFs (Fig. 5G).

DISCUSSION

Appropriate *in vivo* models that can mimic the complex organization of the bone marrow HSC niche have highlighted the fundamental role of BMSCs in the generation and regulation of the hematopoietic microenvironment. Although there is a general consensus that BMSCs have the ability to establish a functional HSC niche, few studies have actually explored whether cells derived from other perinatal or adult tissue sources have a similar capacity (Reinisch et al., 2015). Previously, it has been reported that circulating BBFs with osteogenic character are very rare in mice and humans, but can be routinely found in PB of guinea pigs. Interestingly, some clones of guinea pig BBFs formed cartilage *in vitro*, and formed bone and supported hematopoiesis upon *in vivo* transplantation with a ceramic carrier (Kuznetsov et al., 2001). Here, we report that CB-BFs can support the establishment of BM and hematopoietic niches *in vivo* in pellet-derived ossicles. Similar to BM ossicles, those generated from CB-BFs reproduce the architecture of natural bone (cortical bone, medullary cavity), consisting of donor-derived bone and host-derived sinusoids, and showing the establishment of human CD146$^+$ stromal cells within the BM compartment. However, CB ossicles differed from BM ossicles in terms of dimension and proportion of red and yellow marrow within the BM compartment. Intra-ossicle murine hematopoiesis contained committed lineages, as well as short-term and long-term HSCs in both types of ossicles. In addition, h-CD34$^+$ cells intravenously

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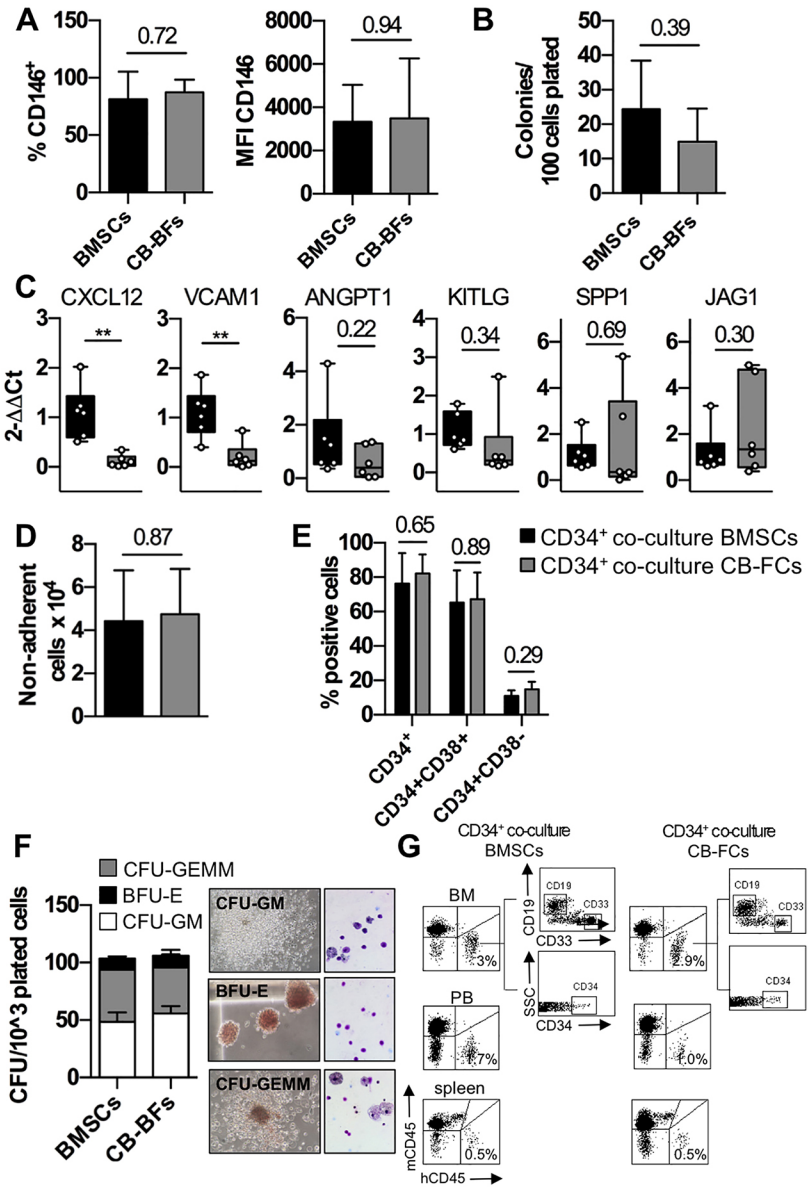

Fig. 5. CB-BFs express CD146 and HSC niche genes and support homeostasis of HSCs *ex vivo*. (A) Expression of CD146 on BMSCs and CB-BFs at passages 3-4 of culture analysed by flow cytometry using anti-human CD146 PE (clone P1H12) (n=3). (B) Colony-forming efficiency (number of colonies initiated by single CFU-Fs) of BMSCs and CB-BFs at passages 3-4 (n=3). (C) Expression of HSC maintenance genes (*CXCL12*, *VCAM1*, *ANGPT1*, *KITLG*, *SPP1* and *JAG1*) in BMSC and CB-BF populations by RT-PCR (n=6). (D) Total number of non-adherent hematopoietic cells harvested after 7 days of co-culture of h-CD34$^+$ cells with CB-BFs or BMSCs (n=3). (E) Percentage of CD34$^+$, CD34$^+$/CD38$^+$ and CD34$^+$/CD38$^-$ subpopulations detected by flow cytometry after 7 days of co-culture of CB-CD34$^+$ cells with CB-BFs or BMSCs (n=3). (F) Clonogenic potential of h-CD34$^+$ cells previously co-cultured with CB-BFs or BMSCs (n=3). (G) Representative analysis of multilineage and multi-organ human chimaerism in NSG mice transplanted with h-CD34$^+$ cells co-cultured on CB-BFs and BMSCs monolayers. P values indicated have been calculated with an unpaired two-tailed t-test.

injected into immunodeficient mice previously implanted with cartilage pellets could stably engraft into the 'humanized niche', as demonstrated by the presence of human HSCs and HSC-derived multilineage reconstitution. These *in vivo* data are correlated with our *in vitro* results, which demonstrate the constitutive expression of HSC maintenance genes in CB-BFs and their capacity to support human HSCs in an *ex vivo* culture system.

Several *in vitro* studies have reported the expression of skeletal markers by CB-BFs exposed to defined stimuli. Furthermore, the successful differentiation of CB-BFs into cartilage in specific chondrogenic medium has been previously demonstrated by our group and by others (Zhang et al., 2011; Pievani et al., 2014; Kern et al., 2006; Jäger et al., 2009; Ragni et al., 2013). In this study, we further showed the expression of known skeletal development regulators by CB-BFs in the absence of any inducing agents. The pattern of expression was similar to that of BMSCs and revealed baseline expression of key genes associated with cartilage and bone formation, including *RUNX2* and *RUNX3*, which are known to be expressed in the growth plate during endochondral bone formation (Wigner et al., 2013). Notably, in a recent study by Reinisch et al., these genes were found to be hypomethylated and overexpressed in BMSCs, but not expressed or only slightly expressed in cell populations isolated from tissue sources other than BM, such as white adipose tissue, umbilical cord and skin (Reinisch et al., 2015). In addition, we formally compared for the first time the chondroid pellets generated by CB-BFs and BMSCs in standard micro-mass cultures. We report that the two types of pellets displayed similar cartilage-like morphology and levels of transcripts for proteoglycans and chondrocyte-associated markers, such as *COL2A1*, *COL10A1*, *SOX9* and *ACAN*. However, the chondroid rudiments generated by CB-BFs were consistently larger than those generated by BMSCs, even though the rate of proliferation and the frequency of precursors were comparable in both specimens, thus suggesting a more efficient production of cartilaginous matrix *in vitro* by the CB-derived progenitors compared with postnatal BMSCs.

In contrast to a large number of *in vitro* investigations, only a few studies have addressed the properties of CB-BFs *in vivo*. Zhang and colleagues were able to demonstrate *in vivo* osteogenesis by CB-BFs and to show the deposition of a large

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amount of newly formed bone in the pores of implanted scaffolds (Zhang et al., 2011). In our recent work, subcutaneous transplantation of CB-BFs in association with a ceramic carrier resulted in the deposition not only of abundant histology-proven bone but also of cartilage. The cartilaginous tissue was formed in the absence of any *ex vivo* induction, thus demonstrating the inherent capacity of CB-BFs to undergo chondrogenesis even in the absence of specific inducing agents (Sacchetti et al., 2016). However, in none of the previous studies was CB-BF differentiation associated with the establishment of a hematopoietic microenvironment (Zhang et al., 2011; Sacchetti et al., 2016). The results presented here provide further elucidation of the biology of CB-BFs, demonstrating that CB-BFs were able to generate complete pellet-derived ossicles *in vivo* in which a functional HSC niche was created. The progressive substitution of cartilage by marrow in the ossicles likely occurred through an 'endochondral myelogenesis' process previously observed in cartilage pellets obtained from BMSCs (Serafini et al., 2014). Interestingly, CB ossicles presented a different ratio of red and yellow marrow compared with the BMSC-derived counterpart, and showed a higher prevalence of red marrow, as demonstrated by histomorphological analyses and by the number of the hematopoietic cells harvested from the transplants. It can be reasonably assumed that this feature of the CB ossicle correlates with the perinatal origin of the donor cells and reproduces the early developmental stages of BM ontogeny. In prenatal life and in infants, in fact, almost the entire bone cavity is filled with red BM, which is then partly replaced by fatty (yellow) marrow with normal maturation (Małkiewicz and Dziedzic, 2012; Laor and Jaramillo, 2009; Bain et al., 2010). Similar to ossicles derived from BMSCs, the hematopoietic tissue within the CB ossicles included mature cells of the erythroid, myeloid and megakaryocytic lineages, as well as blood-cell progenitors, and short-term and long-term HSCs. Moreover, CB-BFs did form a completely humanized hematopoietic niche, in which human HSCs engrafted and were maintained in a functional human microenvironment. Although human ossicles are clearly disadvantaged in their ability to host murine hematopoiesis when compared with femur BM, we did not observe any difference in the engraftment of human cells. One possible explanation is that factors secreted by human stromal cells may not be fully reactive with receptors on murine hematopoietic cells. Moreover, the capacity of the heterotopic medullary cavity to attract human HSCs suggests the establishment of a local cytokine milieu resembling human marrow conditions. Our data indicate that CB-BFs could exert a specific role in promoting migration of HSCs into their BM niche. Further *in vivo* studies to trace the fate of the injected cells will be instrumental to better understand the homing of HSCs to the ossicles and the specific kinetics involved in this process. Importantly, the expression of molecules involved in mobility and cell adhesion, such as CXCL12 and VCAM-1 in CB-BFs (albeit at lower levels, Markov et al., 2007; Wagner et al., 2007) suggests their active role in HSC homing. Moreover, CB-BFs express, like BMSCs, other genes involved in HSC maintenance, such as *ANGPT1*, *KITLG*, *SPP1* and *JAG1*, and support *ex vivo* hematopoietic homeostasis in a co-culture system, providing additional evidence of their capacity to preserve hematopoietic progenitor properties. Taken together, the CB-BFs have many features that are required for functionality in the HSC niche.

The results presented here appear to be in disagreement with our previous study in which bone derived from CB-BFs did not support hematopoiesis (Sacchetti et al., 2016). However, there is a substantial difference in the present study, in that cartilage pellets were obtained through *ex vivo* induction and, subsequently, bone, complete with a medullary cavity, was formed *in vivo* through an endochondral process. Therefore, we can assume that, in the case of CB-BFs, the full expression of an endochondral bone formation program may be required to obtain a marrow niche.

Recently, Reinisch et al. performed a comprehensive comparison of different 'MSC' cell populations and demonstrated that the ability to support the formation of a bone marrow microenvironment is a unique property of BM-derived cells (Reinisch et al., 2015). However, although Reinisch et al. used an experimental model based on an endochondral process, the cells were cultured in a different culture medium (10% human platelet lysate instead of FBS) and were transplanted *in vivo* as undifferentiated cells resuspended in a Matrigel-equivalent matrix. Most importantly, the authors used 'MSCs' from the umbilical cord (UC), which are very different from the fibroblasts derived from CB that we used in this study (Reinisch et al., 2015). In particular, UC cells are not able to form bone *in vivo*, even when transplanted with an osteoconductive carrier (Kaltz et al., 2008; Todeschi et al., 2015). Moreover, significant differences have been found in the global gene expression profile of UC-derived cells and CB-BFs isolated from the same donor and cultured under the same culture conditions (Secco et al., 2009). Taken together, all these differences reinforce the supposition that the UC cell population and CB-BFs population are not the same.

The origin of CB-BFs in blood remaining in the umbilical vein following birth is currently debated. It is possible that these cells are established in the circulation early in prenatal life, as demonstrated by studies on 'MSC' obtained from pre-term cord blood (Campagnoli et al., 2001; Christensen et al., 2004; Surbek et al., 1998; Wyrsch et al., 1999). Owing to the differences between UCs and CB-BFs, it seems very unlikely that CB-BFs detach from the cord and subsequently enter the circulation. Instead, it is possible that these cells, together with HSCs, are travelling, via cord blood, from early fetal hematopoietic sites to the newly formed BM (Tavassoli, 1991; Erices et al., 2000). This suggests that the ontological and anatomical origins of non-hematopoietic cells derived from CB have a profound influence on their differentiation capacities. Our results demonstrate that CB-BFs are a distinct and privileged population of perinatal fibroblasts sharing with other fetal stromal compartments (thymus, spleen and liver) and postnatal BMSCs the extraordinary property of organizing the development of a hematopoietic stem cell niche.

In conclusion, CB-BFs may represent a new and attractive population of skeletal progenitor cells with some advantages over BMSC. First, CB is usually regarded as medical waste and a large amount of samples can be easily obtained at delivery with no risk to the donor. Moreover, as perinatal tissue-derived cells, CB-BFs may have some favourable features that result from their more-primitive ontogenetic origin, including the absence of donor age-dependent variation (Lee et al., 2004). Our data demonstrate that CB-BFs may be used instead of BMSCs in specific experimental settings (e.g. reproduction of bone and the HSC niche at heterotopic sites), and warrant further exploration with the ultimate goal of possible applications in regenerative medicine.

MATERIALS AND METHODS

Cell isolation and cartilage pellet generation

BMSCs were isolated from washouts of discarded BM collection bags of paediatric and adult healthy donors, with informed consent according to institutionally approved protocols, and processed as previously described

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(Gatto et al., 2012). CB-BFs were isolated from umbilical cord blood samples collected from volunteer mothers during elective caesarean sections at term (Pievani et al., 2014). Collections were performed after maternal consent and in accordance with the ethical standards of the San Gerardo (Monza) Hospital ethical committee.

BMSC and CB-BF strains were expanded for two or three passages and then cultured for 3 weeks using a pellet culture system in 15 ml conical tubes at a density of 3×10^5 cells/tube. Cultures were maintained in chondrogenic differentiation medium consisting of DMEM-high glucose (Invitrogen) supplemented with ITS$^+$ premix (BD Biosciences), 1 mM sodium pyruvate (Life Technologies), 50 µg/ml L-ascorbic acid 2-phosphate (Sigma-Aldrich), 0.1 µM dexamethasone (Sigma-Aldrich), 0.1 mM non-essential amino acid solution (Life Technologies) and 10 ng/ml transforming growth factor β1 (TGFβ1) (R&D Systems). At the end of the *in vitro* culture, chondrogenic differentiation was evaluated by histological and molecular analysis.

Quantitative RT-PCR

Total RNA was extracted from cells or cartilage pellets using Trizol reagent (Life Technologies) and cDNA was generated as previously described (Pievani et al., 2014). Quantitative RT-PCR analysis were performed using the ABI 7900 Real-Time PCR system using gene-specific TaqMan Gene Expression Assays and the TaqMan Universal PCR Master Mix (all from Applied Biosystems). TaqMan Gene Expression Assays used are listed in Table S1. Gene expression relative to GAPDH was quantified using the $2^{-\Delta\Delta Ct}$ method.

In vivo transplantation

All animal procedures were approved by the relevant institutional committees. Pellets were transplanted subcutaneously into 8- to 15-week-old female SCID/beige mice (C.B-17/IcrHsd-Prkdc^{scid}Lyst^{bg}, Harlan) ($\leq$16 samples/mouse) and harvested after 8 weeks, as previously described (Serafini et al., 2014).

Histology and histomorphometry

Untransplanted cartilage pellets and heterotopic ossicles harvested at 8 weeks were fixed in 4% formaldehyde in phosphate buffer, decalcified in 10% EDTA, routinely processed for paraffin wax embedding, and used for qualitative and quantitative histological analysis. Serial sections (5 µm) were stained with Haematoxylin and Eosin, Alcian Blue, Toluidine Blue and Picrosirius Red (all from Sigma-Aldrich). Staining with von Kossa was performed on several untransplanted cartilage pellets embedded in paraffin wax without decalcification. For quantitative analysis, a semi-automatic image analyser (IAS 2000, Delta System, Rome, Italy) was used to calculate the area of the untransplanted pellets and, in the heterotopic transplants, the total area of the ossicles and the fraction of BM occupied by adipose tissue.

Immunohistology

For the analysis of cell proliferation, untransplanted cartilage pellets obtained from BMSCs and CB-BFs were immunostained for Ki-67 (clone Mib1, 1:100, Dako, Denmark; DK-2600, Glostrup, Denmark). The colour reaction was developed using 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) as a substrate. Sections were counterstained with Haematoxylin and the fraction of proliferative cells was determined as the number of immunolabelled nuclei/the total of cells counted in the whole section of each pellet.

To establish the contribution of human CB-BFs cells to the generation of the different tissues within the heterotopic ossicles, immunofluorescence experiments were performed using anti-huLaminA/C (EPR4100, 1:500, Abcam) and anti-huCD146 (N1238, 1:25, Novocastra, Leica Biosystems). Alexa Fluor 488-conjugated goat anti-rabbit (A-11008, Invitrogen) and anti-mouse (A-21121, Invitrogen) were used as secondary antibodies for the detection of the binding of anti-huLamA/C and anti-huCD146 antibodies, respectively. Immunolabelled sections were analysed using a Leica TCS SP5 confocal laser scanning microscopy system (Leica Microsystems). Nuclei were stained with TOPRO-3 iodide (Invitrogen).

Bromodeoxyuridine/DNA analysis

BMSCs and CB-BFs at passage 3-4 were cultured for 48 h, incubated with bromodeoxyuridine (BrdU) for 120 min and then harvested, washed and fixed with 70% ice-cold ethanol and kept at 4°C before DNA staining. To detect BrdU incorporated into DNA, fixed cells were incubated with anti-BrdU monoclonal antibody (BD) and total cellular DNA content detected by TO-PRO-3 iodide. Bi-parametric BrdU/DNA analysis was performed on at least 10,000 cells using a FACS Calibur instrument (Becton Dickinson), enabling a clear evaluation of the active S phase cell fraction (BrdU-positive cells).

Evaluation of murine hematopoietic lineages and progenitor cells

Total BM cells were obtained from mouse femurs or the ossicles by flushing and subsequent collagenase digestion using 100 U/ml collagenase type II (Gibco), filtered with 70 µm cell strainers (BD Biosciences) and treated with ammonium chloride solution (Stem Cell Technologies), which lyses red blood cells. To evaluate the presence of different hematopoietic lineages, cells were stained with antibodies against CD45.2APC (clone 104, 17-0454, dilution 1:100), Mac-1PE (M1/70, 12-0112, 1:100), Gr-1FITC (RB6-8C5, 11-5931, 1:250), CD41PE (MWReg30, 12-0411, 1:100) and Ter119PE (TER-119, 12-5921, 1:100).

For evaluation of progenitors and HSCs, cells were incubated with a mouse hematopoietic lineage eFluor450 cocktail (clones 17A2, RA3-6B2, M1/70, TER-119, RB6-8C5, 88-7772, 1:5). Simultaneously, cells were stained with c-kit APC (clone 2B8, 17-1171, 1:100), Sca-1PE (D7, 12-5981, 1:100), CD34 FITC (RAM34, 11-0341, 1:250) and Flk2 PerCP-eFluor710 (A2F10, 46-1351, 1:100) (for HSCs analysis) or FcγR (CD16/CD32) PerCP-eFluor710 (93, 46-0161, 1:100) (for progenitors analysis). All antibodies were from eBiosciences. Analyses were performed using a FACS Canto II instrument with FACS DIVA software (BD).

Murine clonogenic hematopoietic progenitor assay

Hematopoietic clonogenic assays were performed in 35 mm low-adherent surface dishes (Nunc) using 1 ml/dish of MethoCult GF M3434 semisolid medium (Stem Cell Technologies) containing hematopoietic cytokines, according to the manufacturer's instructions. Single-cell suspensions obtained from ossicles, mouse femurs and PB of ossicle-bearing mice were plated in duplicate at 100,000 cells per dish. Colonies were scored after 14 days of incubation based on the morphological criteria as erythroid (BFU-E), granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM), granulocyte/macrophage (CFU-GM), granulocyte (CFU-G) and macrophage (CFU-M).

Hematopoietic reconstitution of ossicle-bearing mice transplanted with human CD34$^+$ cells

CD34$^+$ progenitors from human CB were obtained by Ficoll-Paque Plus (GE Healthcare Europe, Freiburg, Germany) separation of the mononuclear fraction followed by immunomagnetic selection using the CD34 MicroBeads kit (Miltenyi Biotec) [with a median purity of 79% (range, 60 to 90%)].

Three weeks after pellet transplantation, mice were sublethally irradiated (2.5 Gy) using a RADGIL X-Ray treatment unit (Gilardoni, Mandello del Lario, Italy), followed the next day by intravenous injection of 3×10^5 CB-derived human CD34$^+$ cells. Six weeks after transplant of h-CD34$^+$ cells, the engraftment of human cells in PB, spleen, femurs and subcutaneous ossicles was determined by flow cytometry after staining with anti-murine CD45.2 APC and anti-human CD45 PE (clone HI30). Human hematopoietic cells were stained with anti-CD45 (clone 2D1), CD19 (SJ25C1), -CD34 (8G12), -CD33 (P67.6), -CD64 (10.1) and -CD14 (M5E2, all BD Biosciences).

Colony-forming unit-fibroblast assays

The number of clonogenic progenitors at passage 3-4 was determined by the colony-forming unit-fibroblast (CFU-F) assay. Briefly, BMSCs or CB-BFs were seeded at clonal density (1.6 cells/cm^2) and maintained for 14 days in basal medium. To enumerate CFU-F, the cells were fixed with methanol,

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stained with Giemsa solution and scored at 10× magnification. The experiment was performed in triplicate for each sample.

Co-culture of BMSCs/CB-BFs and cord blood CD34$^+$ cells, and *ex vivo* analyses of CD34$^+$ cell homeostasis

CD34$^+$ cells from human CB were obtained as described previously and co-cultured on a confluent layer of BMSCs or CB-FBs in complete RPMI medium (Euroclone) in 12-well plates. Number, phenotype, clonogenicity and engraftment capacity of h-CD34$^+$ cells were assessed after 7 days in culture.

Briefly, non-adherent cells were collected, counted and stained with anti-human CD34 PECy7 and CD38 PE (HIT2, BD Pharmingen). A total of 2×10^3 cells were seeded in MethoCult H4434 semisolid medium (Stem Cell Technologies) following the manufacturer's instructions. Hematopoietic colonies after 14 days of culture were scored under an inverted microscope.

To evaluate the engraftment capacity, 3×10^5 h-CD34$^+$ cells that have been cultured on BMSCs or CB-BFs were transplanted into the tail vein of sublethally irradiated NOD/LtSz-scidIL2R$\gamma^{-/-}$ (NSG) mice. Mice were euthanized 4 weeks after transplantation to evaluate human chimaerism.

Statistical analysis

Data are plotted as mean±s.d.; *n* is indicated in figure legends. Statistical analyses were performed using an unpaired two-tailed Student's *t*-test. A *P*-value of less than 0.05 was considered to be statistically significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization, M.R. and M.S.; Methodology, A.P., B.S., B.R., S.D., V.S. and C.R.; Investigation, A.P., B.S. and A.C.; Writing - Review and Editing, P.V., A.B. and P.G.R.; Supervision, M.R. and M.S.; Funding Acquisition, A.B., P.G.R., M.R. and M.S.

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Supplementary information

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