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Establishment of bone marrow and hematopoietic niches in vivo by reversion of chondrocyte differentiation of human bone marrow stromal cells

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Abstract Human bone marrow stromal cells (BMSCs, also known as bone marrow-derived "mesenchymal stem cells") can establish the hematopoietic microenvironment within heterotopic ossicles generated by transplantation at non-skeletal sites. Here we show that non-mineralized cartilage pellets formed by hBMSCs ex vivo generate complete ossicles upon heterotopic transplantation in the absence of exogenous scaffolds. These ossicles display a remarkable degree of architectural fidelity, showing that an exogenous conductive scaffold is not an absolute requirement for bone formation by transplanted BMSCs. Marrow cavities within the ossicles include erythroid, myeloid and granulopoietic lineages, clonogenic hematopoietic progenitors and phenotypic HSCs, indicating that complete stem cell niches and hematopoiesis are established. hBMSCs ($CD146^+$ adventitial reticular cells) are established in the heterotopic chimeric bone marrow through a unique process of endochondral bone marrow formation, distinct from physiological endochondral bone formation. In this process, chondrocytes remain viable and proliferate within the pellet, are released from cartilage, and convert into bone marrow stromal cells. Once explanted in secondary culture, these cells retain phenotype and properties of skeletal stem cells ("MSCs"), including the ability to form secondary cartilage pellets and secondary ossicles upon serial transplantation. Ex vivo, hBMSCs initially induced to form cartilage pellets can be reestablished in adherent culture and can modulate gene expression between cartilage and stromal cell phenotypes. These data show that so-called "cartilage differentiation" of BMSCs in vitro is a reversible phenomenon, which is actually reverted, in vivo, to the effect of generating stromal cells supporting the homing of hematopoietic stem cells and progenitors.

Introduction

Almost everything significant that we know about the biology of bone marrow stromal cells [also known as bone marrow-derived "mesenchymal stem cells" ("MSCs")] comes

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from the use of heterotopic transplantation systems. Over the past 45 years, progressive refinements of a single seminal experiment (transplantation of bone-less fragments of bone marrow) (Tavassoli and Crosby, 1968), have led in a stepwise manner to the identification of "MSCs," first as non-hematopoietic stromal cells (bone marrow stromal cells, BMSCs) [reviewed in Friedenstein, 1990], then to a subset thereof noted for the ability to initiate clonal growth in vitro (colony forming unit-fibroblast, CFU-F) (Friedenstein et al., 1970), then to single multipotent clonogenic progenitor (a skeletal stem cell) (Friedenstein et al., 1970; Kuznetsov et al., 1997), and ultimately to a perisinusoidal adventitial cell (adventitial reticular cell/pericyte), amenable to prospective isolation (Sacchetti et al., 2007). In all of these cases, formation of a complete heterotopic ossicle featuring bone and bone marrow has been the fundamental readout of the experiments, which: i) revealed the existence of a skeletogenic potential in bone marrow, ii) ascribed it to stromal cells, iii) identified multipotent progenitors, and iv) ultimately provided evidence for self-renewal and in situ identity of the long-postulated, bona fide stem cell for skeletal tissues that was at one point inappropriately renamed "mesenchymal stem cell" [reviewed in Bianco et al., 2013].

Different versions of the heterotopic transplantation assays are currently in use, which differ from one another with respect to the site of grafting (kidney subcapsular space, subcutaneous tissue) or type of scaffold/material employed as a carrier, which is a prerequisite for use of human cells (Krebsbach et al., 1997). Methods for studying human BMSCs by in vivo transplantation require the use of mineralized, osteoconductive scaffolds (Adachi et al., 2005; Krebsbach et al., 1997; Sacchetti et al., 2007). Currently, the same basic principle used for generating experimental transplants in immunocompromised mice is directly translated into protocols designed for human BMSC-directed bone regeneration in the clinic (Mankani et al., 2001; Mankani et al., 2008; Quarto et al., 2001). Thus, a variety of ceramic scaffolds are regarded as necessary and useful for harnessing the ability of BMSCs to generate histology-proven bone in vivo, when specifically dealing with human rather than murine stromal progenitors (Krebsbach et al., 1997).

A subset of human BMSCs appears to coincide with a perisinusoidal population of clonogenic stromal cells noted for the expression of CD146, CD105, and ALP (Sacchetti et al., 2007). When cultured as non-differentiated cells, loaded onto an osteoconductive scaffold, and then transplanted in vivo, these cells are able to transfer and organize, at heterotopic sites, a hematopoietic microenvironment. This event follows the establishment of heterotopic bone and a sinusoidal network, and coincides with the establishment of a perisinusoidal compartment of $CD146^+$ human stromal cells (Sacchetti et al., 2007). These data, and similar data later obtained with mouse BMSCs (Mendez-Ferrer et al., 2010) have put BMSCs at center stage in the search for the hematopoietic "niche"-maintaining cells. As a result, the hematopoietic stem cell niche is currently seen as the site of a unique interplay of two different stem cells (Bianco, 2011; Mendez-Ferrer et al., 2010; Sacchetti et al., 2007).

While classical, the use of heterotopic transplantation systems for investigating the biology of the hematopoietic microenvironment holds new promises as a result of these recent data and views. However, to maximize the value of in vivo transplantation in order to further elucidate the role of BMSCs in the HSC niche, systems that facilitate the ex vivo characterization of stromal and hematopoietic cell populations within the heterotopic bone marrow are highly desirable. While more refined formulations of mineralized scaffolds (such as different proportions of HA/TCP) are developed that may allow for ultimate complete removal of the scaffold long term, remnants of these materials are commonly seen in experimental transplants in immunocompromised mice, typically harvested at 8 weeks. This prevents establishment of a completely normal architecture of bone and marrow, and it confounds and complicates the analysis of cell populations contained within the ossicle, as well as the use of proper quantitative assessments of pre- and post-grafting cell populations and whole tissue components due to difficulties in quantitatively liberating cells from inert ceramic scaffoldings.

Using a transplantation system free of endogenous scaffolding, we show here that in vivo generation of a human hematopoietic microenvironment (HME)/niche does not require transplantation of undifferentiated cells: by transplanting unmineralized cartilage pellets obtained ex vivo from human BMSCs, a complex developmental cascade is initiated, which is sharply distinct from endochondral ossification. In this system, chondrocytes revert to a stromal cell phenotype, and establish the HME/niche. Neither partner of the dual stem cell bone marrow niche (skeletal and hematopoietic) is actually transplanted in this system: donor skeletal stem cells are generated (locally and in vivo) from transplanted chondrocytes, while host (murine) hematopoietic stem cells and hematopoietic progenitors are recruited from the circulation.

Materials and methods

Cell isolation and culture

Total nucleated cells were isolated from the washouts of discarded bone marrow collection bags and filters used for BM transplantation, with informed consent per institutionally approved protocols. Cells were derived from healthy pediatric (n = 9, aged from 9 months to 12 years) and adult donors (n = 7, aged from 17 years to 44 years) and processed as previously described (Sacchetti et al., 2007). Human BMSC (hBMSC) populations (at the 2nd or 3rd passage) were grown for 3 weeks as unmineralized pellets in 15 ml polypropylene conical tubes at a density of 3×10^5 cells/tube in Chondrogenic Differentiation Medium [CDM; DMEM-High glucose (Invitrogen, Carlsbad, CA) supplemented with ITS™ premix (BD Biosciences, San Jose', CA, USA), 1 mM pyruvate (Sigma-Aldrich, St. Louis, MO), 50 μg/ml 2-phosphate–ascorbic acid (Fluka, Sigma-Aldrich), 100 nM dexamethasone (Sigma-Aldrich), and 10 ng/ml TGFβ1 (R&D systems, Minneapolis, MN) or 10 ng/ml TBFβ3 (Sigma-Aldrich)]. To induce mineralization for specific experiments, pellets were cultured for 3 weeks in CDM followed by 2 weeks in medium supplemented with 7.0×10^{-3} M β-glycerophosphate and 50 nM thyroxine (without TGFβ1) (Muraglia et al., 2003). At the end of the in vitro

culture, chondrogenic differentiation was evaluated by histology, immunohistology and q-RT-PCR analysis. For specific experiments, cartilage pellets were collagenase-digested and the resulting cell suspensions used as further described. In some cases, pellets, unmineralized or mineralized, were irradiated (30 Gy, 2.42 Gy/min) prior to transplantation.

In vivo transplantation

All animal procedures were approved by the relevant institutional committee. Pellets were transplanted subcutaneously into 8–15-week old female SCID/beige mice (C.B-17/IcrHsd-Prkdc^{scid}Lyst^{bg}, Harlan, Inc., Indianapolis, IN) ($\leq$16 samples/mouse) and harvested at different time points, essentially as described for conventional BMSC-scaffold-containing constructs (Krebsbach et al., 1997; Sacchetti et al., 2007).

Radiography

Untransplanted cartilage pellets or harvested heterotopic transplants were exposed to X-rays at 20 kV for 5 s in a Faxitron MX-20 X-ray machine (Faxitron X-Ray Corp., Buffalo Groove, IL) with Kodak MIN-R mammography film (Carestream Health, Rochester NY, USA). Magnification used was at 5×.

Histology and immunohistology

Untransplanted cartilage pellets or heterotopic transplants were harvested, fixed in 4% formaldehyde in phosphate buffer, decalcified in 10% EDTA and processed for paraffin embedding, or alternatively, samples were snap-frozen in OCT embedding medium in liquid nitrogen and cryostat-sectioned serially. Five micrometer thick sections were stained with hematoxylin and eosin (Sigma-Aldrich), Safranin O-Light Green or Alcian Blue (Sigma-Aldrich). For von Kossa staining, samples were embedded undecalcified in glycol methacrylate, and the staining was performed on 2–4 μm thick sections (Bianco et al., 1984). Primary antibodies used for immunolocalization studies as per established protocols are listed in Table S1. Brightfield light microscopy images were obtained using a Zeiss Axiophot epifluorescence microscope (Carl Zeiss, Germany). For confocal fluorescence microscopy (Robertson et al., 2008), immunolabeled sections were analyzed using a Leica TCS SP5 confocal laser scanning system (Leica Microsystems, Mannheim, Germany). Secondary antibodies, Alexa Fluor 594 and 488, were from Molecular Probes (Invitrogen). Nuclei were stained by TOPRO3 (Invitrogen). In Situ cell death detection kit (TUNEL) with fluorescein-conjugated antibodies was from Roche Diagnostics GmbH (Mannheim, Germany).

Secondary culture of cells from untransplanted pellets

Cartilage pellets were washed in PBS and digested twice with 100 U/ml type II collagenase (Gibco, Grand Island, NY) in PBS with 3 mM $CaCl_2$ for 1 h at 37 °C. 2 × 10^5 cells obtained from the two digestions of pooled pellets (n = 9–16) were used for FACS analysis for human CD146 expression. The remaining cells were seeded at clonal density (<1.6 cells/cm^2) and colony formation (>50 cells/colony) was scored at 2 weeks. Colonies surrounded by cloning cylinders were trypsin-released and analyzed by FACS for expression of human CD146. When relevant, cell suspensions obtained by collagenase digestion of pellets were magnetically depleted of the human CD146$^+$ fraction using MidiMACS (Miltenyi Biotech, Bergisch Gladbach, Germany); the resulting human CD146$^-$ fraction was used for the colony forming efficiency assay, and other in vitro assays. Multi-colony populations and single colonies were harvested at 2 weeks, and analyzed for human CD146 expression by FACS analysis.

RNA isolation and q-RT-PCR reaction

Total RNA was extracted using TRIZOL® reagent (Invitrogen), following the manufacturer's instructions. 1 μg of RNA was then reverse transcribed with the use of a SuperScript® II Reverse Transcriptase kit (Invitrogen) in the presence of random hexamers. q-RT-PCR reaction assays were performed in triplicate on an ABI 7900 Real-Time PCR system thermal cycler with the qPCR Mastermix (Applied Biosystems-Invitrogen). All TaqMan Gene Expression assays were provided by Applied Biosystems (Table S2). The relative expression of each gene was normalized to the reference gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Total mRNA levels were quantified using the comparative threshold cycle method.

Secondary passage, secondary differentiation, and secondary transplant of ossicle-derived stromal cells

After 8 weeks in vivo, heterotopic transplants were harvested and collagenase-digested and stromal cells present were isolated by plastic adhesion. Stromal cells obtained after 2–3 weeks of culture, were characterized for the expression of typical human BMSC markers and cultured as micromasses under previously described chondrogenic conditions. Cartilage pellets obtained were either analyzed histologically, or implanted in immunocompromised mice for 8 weeks and then harvested for histology.

Flow cytometry

After 8 weeks in vivo, heterotopic transplants were harvested and digested with type II collagenase and single cell suspensions made as described above. The cell suspension was treated with red cell lysis buffer (except for cells used for Ter119 staining) and stained with fluorochrome-labeled monoclonal antibodies listed in Table S3. To identify hematopoietic lineage negative cells, mouse Hematopoietic Lineage Cocktail^{eFluor450} (from eBioscience, San Diego, CA) was used. Analyses were performed using a FACS Canto II instrument and FACS DIVA software (BD Biosciences, Franklin Lakes, NY). For studies on the heterotopic bone marrow stroma, cells were stained with PE-labeled antibodies against human antigens listed in Table S3. Flow cytometric analysis was performed on 10,000 events with the use of a FACSCalibur cytometer and data were analyzed using the CellQuest PRO software (BD Biosciences). The appropriate isotype-matched antibodies were used as negative controls in all cases.

Hematopoietic colony-forming efficiency (h-CFE) assay

The h-CFE assay was performed in semi-solid medium supplemented with hematopoietic cytokines. Briefly, harvested heterotopic ossicles were digested and filtered to obtain single-cell suspensions. Cells were resuspended in 1 ml of MethoCult GF M3434 (StemCell Technologies, Vancouver, BC), plated in 35 mm low-adherence plastic dishes (Nunc, Rochester, NY), and incubated at 37 °C and 5% CO_2. Hematopoietic colonies were identified by morphology on an inverted microscope. The nature of individual colonies was confirmed by plucking colonies, cytospinning the cells on glass slides, and staining with May-Grunwald Giemsa.

Statistical analysis

Student's paired *t* test was used for statistical comparisons between groups. P values less than 0.05 were considered statistically significant.

Results

BMSC-derived chondroid pellets can generate heterotopic ossicles with the natural architecture of the bone/bone marrow organ

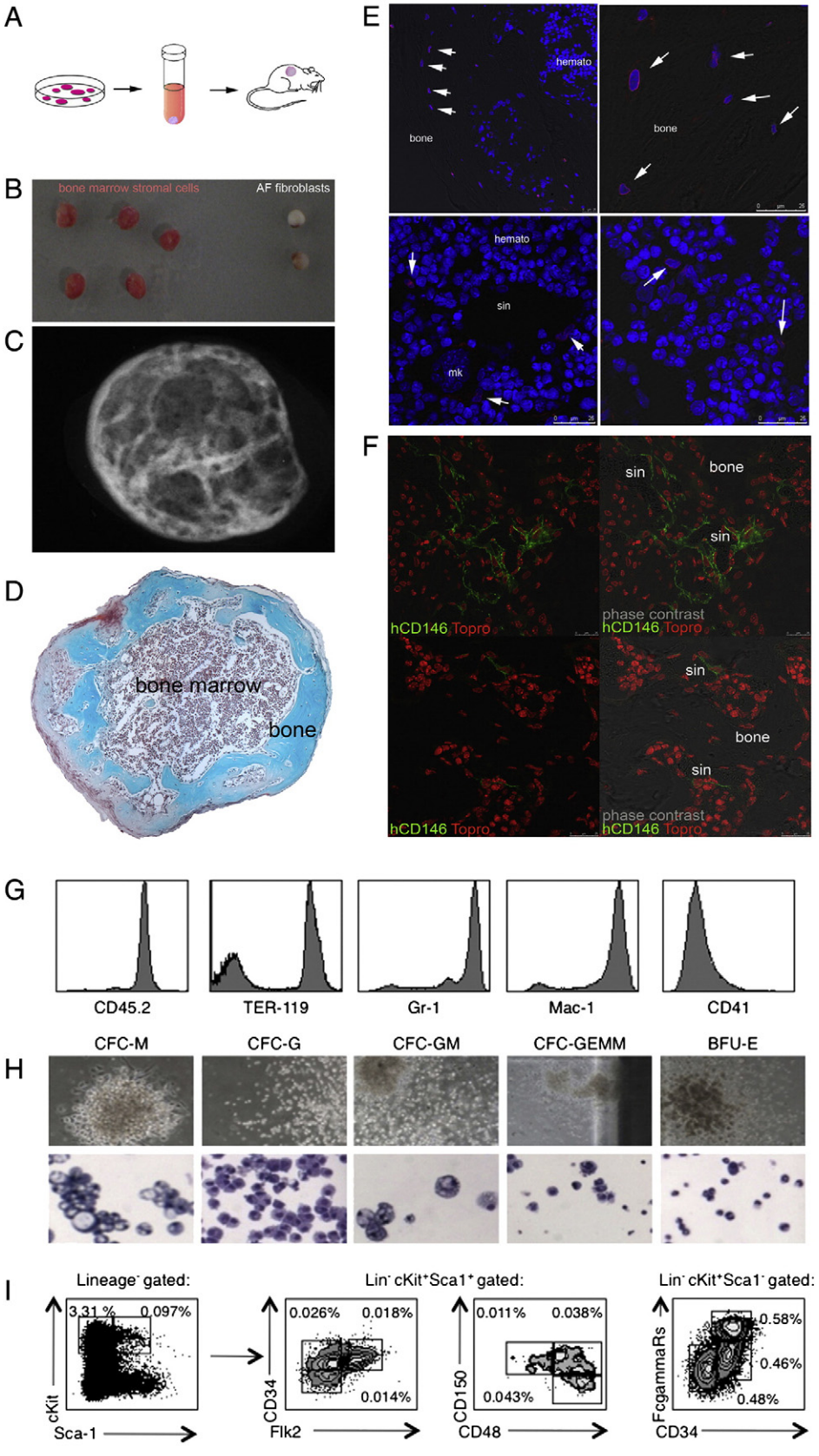
To generate heterotopic ossicles without the addition of an exogenous scaffold, we established pellet cultures with hBMSCs (3×10^5 cells per pellet), and incubated them with 10 ng/ml of TGFβ1 or TGFβ3 for 3 weeks prior to in vivo transplantation into the subcutaneous tissue of immunocompromised mice (Fig. 1A, Fig. S1). At the end of the culture period, cartilage pellets obtained with either TGFβ1 or TGFβ3 were essentially identical to one another, and devoid of any mineral as assessed by either von Kossa staining or Faxitron™ analysis (data not shown). X-ray and histological analysis of grafts harvested at 8 weeks (n = 217, from 8 hBMSC donors) demonstrated that complete heterotopic ossicles (i.e., structures including bone and bone marrow) had developed in vivo (Figs. 1B–D). Each pellet, and each resulting ossicle was generated by as little as 1/7th the number of hBMSCs as typically used in a exogenous scaffold-based transplant (3×10^5 vs. 2×10^6 cells). At variance with ossicles that develop from conventional transplants of human cell-material constructs, pellet-derived ossicles demonstrated a striking architectural resemblance to natural bone/marrow organs; i.e., they featured a regular bone cortex encasing a marrow cavity filled with hematopoietic tissue, including systems of marrow sinusoids (Fig. 1D). Bone cells were human in origin throughout, as demonstrated by nuclear immunoreactivity for human-specific Lamin A/C (>95% of osteocytes and osteoblasts; Fig. 1E); hematopoietic cells and sinusoidal endothelium were murine (Fig. 1E), and a distinct population of human adventitial reticular cells, immunoreactive for human CD146, covered the abluminal surface of sinusoids (Fig. 1F). Hematopoietic tissue within the ossicle included erythroid, myeloid and megakaryocytic lineages (Fig. 1G), as well as hematopoietic clonogenic progenitors assayable in methylcellulose (Fig. 1H). Immunophenotypic ST-HSC (LSK $Flk2^-CD34^+$, 0.029 ± 0.004%, n = 20) and LT-HSC (LSK $Flk2^-CD34^-$, 0.012 ± 0.004%, n = 20) (Adolfsson et al., 2001) were readily detectable in the ossicles. Within the $Lin^-/c\text{-}Kit^+/Sca\text{-}1^-$ fraction, phenotypic MPP progenitors (common myeloid progenitors, CMPs — $FcR^{lo}CD34^+$; megakaryocyte/erythrocyte lineage-restricted progenitors, MEPs — $FcR^{lo}CD34^-$; granulocyte/macrophage lineage-restricted progenitors, GMPs — $FcR^{hi}CD34^+$) (Akashi et al., 2000), were also identified (Fig. 1I).

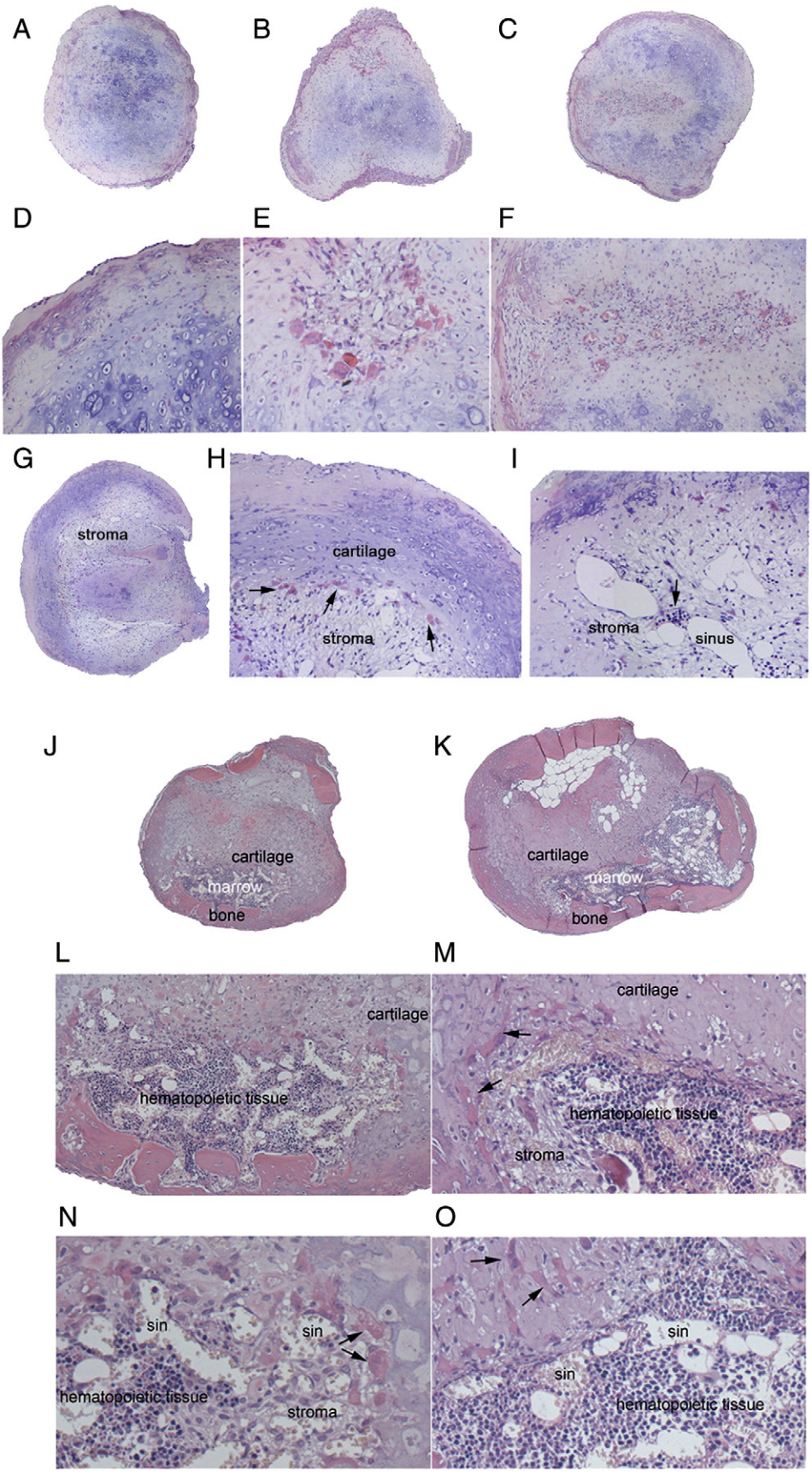
Pellet-derived ossicles are generated through a process distinct from physiological endochondral ossification

To elucidate how such striking architectural mimicry of the natural bone/bone marrow organ was established, we analyzed the development of ossicles in serially harvested transplants. As demonstrated by von Kossa staining and Faxitron™ analysis, cartilage was entirely unmineralized at the end of the culture period and prior to transplantation. However, partial mineralization of the transplanted cartilage pellet was detectable as early as 3 weeks post-transplantation (Figs. 2A, D). Concurrently, mineralized

Figure 1 Chondroid pellets made from hBMSCs generate complete ossicles when transplanted in vivo. A) Scheme of the transplantation protocol. Multicolony-derived strains of hBMSCs are induced to formation of cartilage by culturing as pellets in the presence of TGFβ1 for 3 weeks. Pellets are then transplanted into the subcutaneous tissue of immunocompromised mice to generate heterotopic ossicles. B) Gross appearance of 5 heterotopic ossicles (from 2 donors) harvested at 8 weeks, and 2 identically processed control transplants made with pellets generated from amniotic fluid fibroblasts. The vivid red color of hBMSC-generated ossicles reflects their content in hematopoietic tissue. C) Representative FAXITRON radiogram of a heterotopic ossicle at 8 weeks. Note the shell of cortical bone and the inner system of bone trabeculae (radio-opaque), and multiple coalescent marrow cavities (radio-lucent). D) Histology of a heterotopic ossicle demonstrating cortical bone (bone) and a marrow cavity populated with hematopoietic cells (bone marrow). (Safranin O-Light Green staining.) E) Human origin of the heterotopic bone, as demonstrated by expression of Lamin A/C, localized to the inner aspect of the nuclear membrane of osteocytes in confocal fluorescence images. Hematopoietic cells populating the marrow space are murine in origin; however, single human nuclei (arrows) are found scattered among mouse hematopoietic cells (mk, megakaryocyte.) F) Confocal fluorescence images demonstrating bone marrow stromal cells expressing hCD146 in the heterotopic bone marrow. Images on the right have superimposed phase contrast images to visualize red blood cells in sinusoids (sin) as well as bone. Note the adventitial position of hCD146-expressing stromal cells, and their branched morphology (adventitial reticular cells.) G) Heterotopic ossicles are colonized by multilineage murine hematopoietic cells and progenitors. Expression of hematopoietic markers in heterotopic ossicles. H) Morphology of colonies and cells formed by heterotopic ossicle-derived cells in methylcellulose at day 14. I) Evaluation of the presence of LT-HSC, ST-HSC and MPP progenitors (GMP, CMP and MEP) by FACS analysis.

cartilage was focally being resorbed by osteoclasts (Figs. 2B, E), while unmineralized cartilage was being invaded by blood vessels (Figs. 2C, F). The latter process was directly reminiscent of the formation of epiphyseal vascular canals in fetal bone [which involves MT1-MMP-mediated, osteoclast-free degradation of unmineralized cartilage (Holmbeck et al.,

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1999)]. Whether mineralized or unmineralized, the cartilage was progressively replaced, in the absence of local bone formation, by a primitive, vascularized stromal tissue, which then became colonized by hematopoietic cells (Figs. 2G–I). Sites of bone formation and cartilage resorption remained spatially segregated from one another within the developing ossicle (Figs. 2J–L), at striking variance with the natural process of endochondral ossification. Indeed, at no time point post-transplantation did bone in the ossicle include a mineralized cartilage core, which would mark and denote a true endochondral process of ossification. In partially developed ossicles, cortical bone and resorbing cartilage formed opposite boundaries of the developing marrow, which was "sandwiched" in between (Figs. 2J–O). Hence, the transition from cartilage pellets to heterotopic ossicles was significantly different from the natural process of endochondral ossification as commonly known. It consisted of the replacement of cartilage by marrow (via a stromal intermediate) rather than bone, and could be best denoted as a unique process of "endochondral myelogenesis," as distinct from endochondral ossification.

Survival and proliferation of chondrocytes are required for the development of heterotopic bone marrow (endochondral myelogenesis)

During the normal process of endochondral ossification in vivo, chondrocytes traverse a sequence of events; i.e., proliferation, proliferation arrest, hypertrophy and apoptosis, which precedes the removal of mineralized cartilage and its replacement by new bone (Calvi et al., 2003; Kronenberg, 2003). As demonstrated by TUNEL staining, no apoptosis of chondrocytes occurred within transplanted cartilage undergoing conversion into heterotopic ossicles (Figs. 3A,B). Ki67 labeling in pellets harvested at 4–6 weeks revealed that ~5% of the chondrocytes were proliferating within the cartilage pellet in vivo (Fig. 3C). Concurrent with the ongoing replacement of cartilage by marrow, proliferating human stromal cells ($CD146^{+}$) accumulated adjacent to the cartilage resorption front (Fig. 3C, D), and an adventitial position at the wall of blood vessels (Fig. 3E).

In vivo proliferation of chondrocytes did not translate into growth of the ossicle relative to the originally transplanted pellets, and cartilage itself was progressively replaced by marrow over time (Figs. 3F–I). To determine if chondrocyte growth had any functional bearing on the development of the bone marrow, we analyzed the effects of arresting proliferation of chondrocytes prior to transplantation. To this end, cartilage pellets were either irradiated, or induced to hypertrophy and mineralize [by subculturing for two additional weeks under mineralizing conditions, as described (Muraglia et al., 2003)], or both, prior to transplantation (Figs. 4A–F). The relative volumes of cartilage and marrow were then measured in grafts harvested at 8 weeks (Fig. 4C). Induction of ex vivo mineralization significantly reduced the efficiency of conversion of the cartilage pellet into a complete heterotopic ossicle (bone with a bone marrow cavity; Figs. 4A–C). No marrow developed in pellets (either unmineralized or mineralized) that were irradiated prior to transplantation (Figs. 4D–E). Cartilage mineralization in vivo was per se unaffected in irradiated pellets that were not induced to mineralize in vitro; formation of an outer shell of cortical bone was also not affected (Fig. 4F). Therefore, the ability of chondrocytes to proliferate in vivo was required for the in vivo development of a heterotopic marrow, but not of heterotopic bone.

Human bone marrow stromal cells emerge from cartilage in vivo

Because stromal cells are the only component of the heterotopic marrow contributed by the transplanted human cells, these data suggest that chondrocytes could contribute to the establishment of the human stroma in the heterotopic marrow in vivo. To evaluate this possibility, we analyzed by confocal fluorescence microscopy the histological events taking place at the site of emergence of the newly formed stroma; i.e., at the cartilage resorption front of developing ossicles. Human CD146 [a known marker of human stromal cells that is not expressed in chondrocytes (Sacchetti et al., 2007)] and Col2 provided convenient markers to this end (Figs. 5A–E). Chondrocytes adjacent to the front of cartilage resorption expressed clearly detectable membrane-associated human CD146 (Figs. 5B–E). Stromal cells at the marrow side of the cartilage resorption front also expressed human CD146 (Figs. 5A–E), with distinctly higher levels of fluorescence intensity ($hCD146^{hi}$) compared to "frontline" chondrocytes ($hCD146^{low}$). Chondrocytes situated deeper, within the cartilage side of the resorption front, did not express human CD146 ($hCD146^{-}$). At the resorption front, chondrocyte lacunae were breached by resorbing osteoclasts [clearly visualized by autofluorescence in confocal images (Figs. 5A,D)], making a pathway to the adjacent marrow space for the viable chondrocytes at the resorption "frontline." Taken together, these observations suggested that replacement of cartilage by marrow was associated with the local emergence of CD146-expressing stromal cells directly from the chondrocytes, as a result of survival, modulation of phenotype, and local migration of the cells upon the resorption of the surrounding cartilage matrix.

Figure 2 Developmental stages of heterotopic ossicles. A–C) Submacroscopic views of cross-sections of pellets harvested at 20 days post-transplantation. The bulk of the tissue is still cartilage, and there is no bone marrow. However, a mineralized phase is seen at the periphery (D) as confirmed by von Kossa staining (not shown); osteoclastic resorption through mineralized sites (E), and vascular invasion of unmineralized sites (F) have begun, and a primitive stroma appears at both types of sites. G) Submacroscopic view of a pellet harvested at 40 days. An extensive portion of cartilage has been replaced by primitive stromal tissue (stroma). H) Osteoclasts (arrows) resorb mineralized cartilage, which is replaced by stromal tissue (stroma). I) The stromal tissue includes sinusoid-type vessels, around which nascent hematopoietic foci are seen (arrow). J,K) Ossicles harvested at 60 days, sectioned through areas of persisting cartilage and newly formed bone marrow. This is comprised between cortical bone and cartilage, is enriched in hematopoietic cells, and has a fully developed system of sinusoids. L,O) Cartilage is undergoing osteoclastic resorption (arrows), and osteoclasts penetrate through cartilage (arrows in O); resorption of cartilage is not coupled to deposition of new bone, but is associated with the appearance of stromal tissue (stroma).

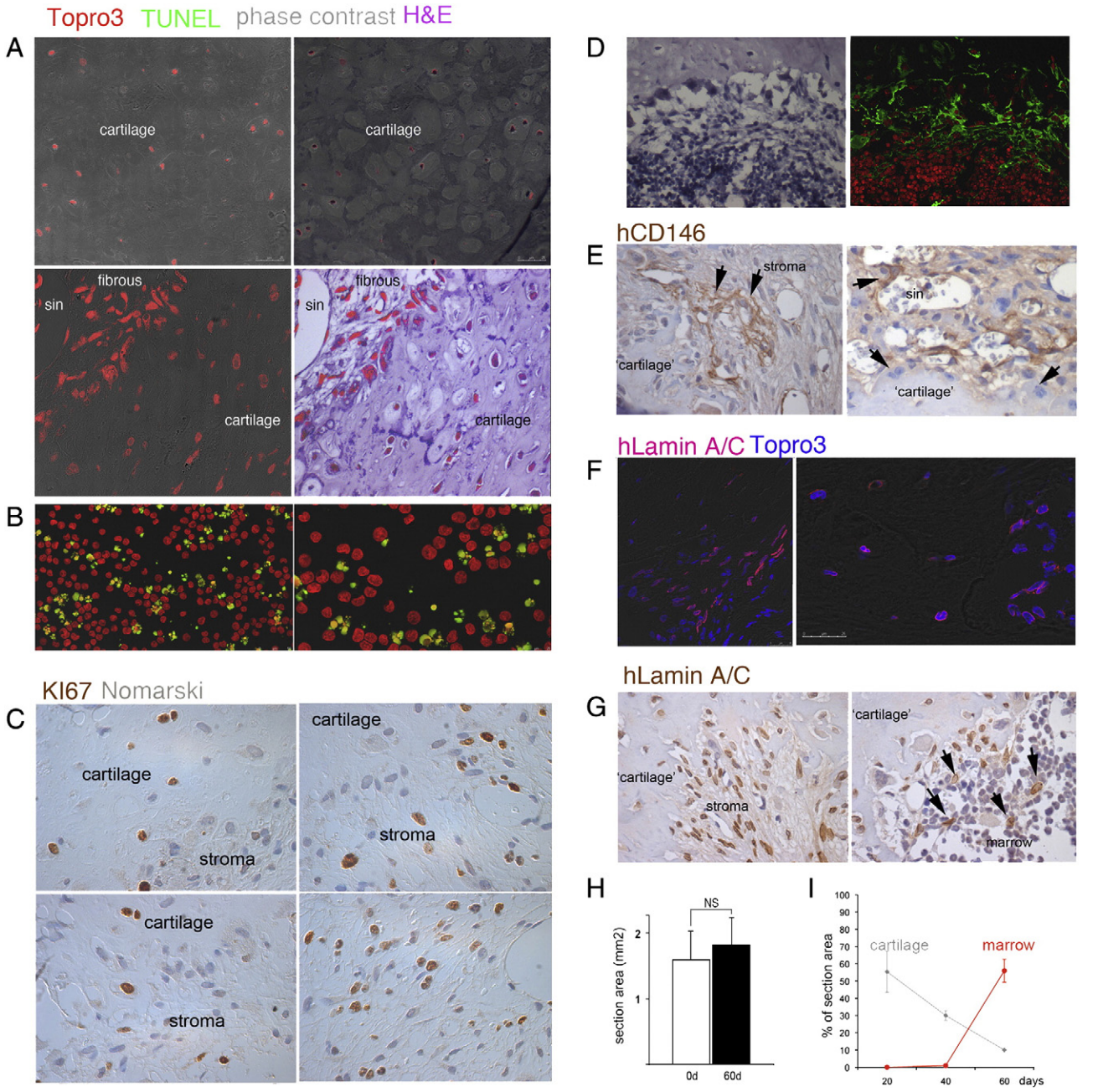


Figure 3 Survival and proliferation of chondrocytes, and generation of human bone marrow stroma within in vivo remodeling pellets. A) Lack of TUNEL staining in chondrocytes at the cartilage/marrow interface. B) TUNEL positive control. C) Ki67 labeling in chondrocytes and adjacent nascent stroma. D) Newly formed stromal cells expressing hCD146 at the resorbing cartilage boundary. Left and right, same field viewed in transmitted light, H&E staining, and confocal immunofluorescence. E) Perisinusoidal stromal cells expressing hCD146 at the resorbing cartilage boundary, transmitted light immunoperoxidase. F) Lamin A/C immunolabeling of newly formed human stroma next to resorbing cartilage. G) Lamin A/C immunolabeling of newly formed human stroma next to resorbing cartilage, transmitted light immunoperoxidase. Left, boundary between cartilage and stroma; right, boundary between cartilage and hematopoietic marrow. Arrows point to human stromal cells around sinusoids. H) Size of pellets and resulting ossicles. Section area of whole grafts before transplantation and after 8 weeks in vivo, n = 4. I) Growth of marrow relative to cartilage. Percentage of section area occupied by cartilage and marrow, n = 4 per time point.

CD146$^+$ stromal cells can be generated by CD146$^-$ "chondrocytes" ex vivo

This implied that once BMSCs differentiated into "chondrocytes" within a pellet ex vivo, they could still revert to a stromal phenotype. To investigate this possibility further, we first analyzed CD146 expression, vis-à-vis the expression of cartilage markers [*COL2*, aggrecan (*ACAN*), *SOX9*], in non-differentiated human CD146$^+$ stromal cells and in pellets generated ex vivo from parallel cultures from the same donors (Fig. 6A). Similar

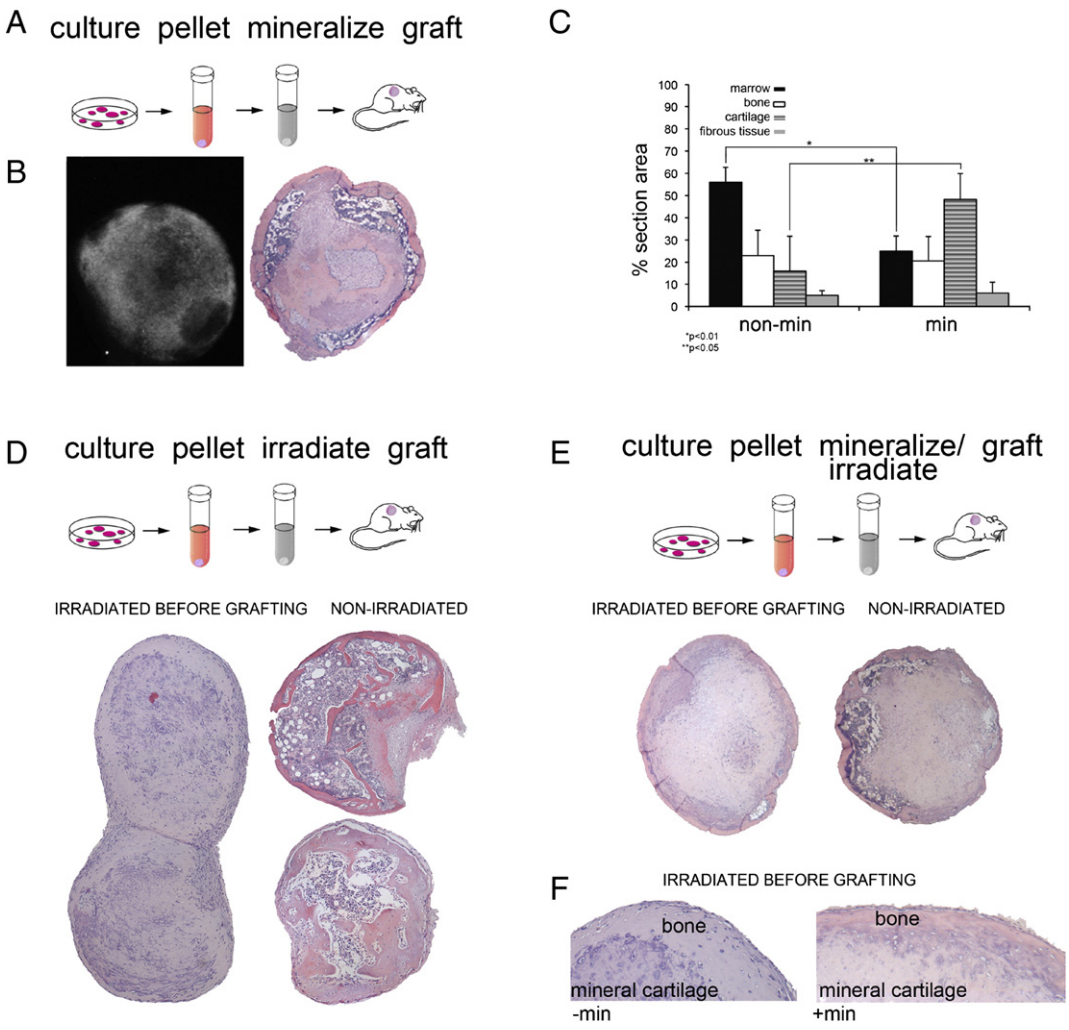


Figure 4 Matrix mineralization or arrested proliferation of chondrocytes impair remodeling of cartilage into marrow. A) Pellets cultured for 3 weeks under chondrogenic conditions were subcultured under mineralizing conditions for 2 further weeks prior to transplantation. B) Left, X-ray of ossicle harvested at 8 weeks demonstrating a diffuse pattern of mineralization (compare with Fig. 1), lack of a trabecular structure, and an abortive marrow cavity. Right, histology of the same ossicle demonstrating abundant residual mineralized cartilage, and limited amounts of bone marrow. C) Histomorphometry of relative amounts of different tissues in ossicles made from pellets that were or were not induced to mineralize prior to transplantation. Significantly more marrow and less cartilage are observed in pellets that are transplanted unmineralized. D) Irradiation prior to transplantation of non-mineralized pellets blocks ossicle formation. Note the absence of any bone marrow in irradiated pellets. E) Irradiation prior to transplantation of ex vivo mineralized pellets blocks the formation of marrow. No marrow is seen in the irradiated pellet. F) Irradiation of either unmineralized or mineralized pellets prior to transplantation does not prevent mineralization of cartilage or deposition of cortical bone.

analyses were conducted on intact cartilage pellets and on cultures established by digesting pellets generated in the same experiments and established in vitro (Fig. 6B). Quantitative RT-PCR analysis demonstrated that CD146 mRNA was highly expressed in primary BMSC cultures, and markedly down-regulated during ex vivo chondrogenesis, while canonical cartilage markers were concurrently upregulated as expected (Fig. 6A). A reverse change in pattern of marker expression was observed in cultures established by cells liberated from the pellet compared to intact pellets (Fig. 6B). From cultures of pellet-derived adherent cells, cartilage differentiation was efficiently obtained upon secondary pellet culturing. In intact pellets, CD146-expressing cells could not be demonstrated by immunocytochemistry in untransplanted, undigested pellets, in which all canonical cartilage markers (COL2, SOX9, ACAN) could otherwise be readily demonstrated at the protein level (not shown). However, FACS analysis of cell suspensions obtained from digested pellets revealed low levels of expression (low counts, low MFI) of CD146 (Fig. 6C), raising the possibility that a few non-differentiated cells could be retained in the pellet and re-established in culture as stromal cells. However, $CD146^{-}$ cells sorted from collagenase-digested untransplanted pellets adhered to plastic, and turned on CD146 expression at high levels within less than 2 weeks. Interestingly, a high proportion of pellet-derived $CD146^{-}$ cells formed discrete colonies at clonal density, all of which were intensely and uniformly $CD146^{+}$ (Fig. 6D). This demonstrated that pellets contained clonogenic $CD146^{-}$ cells giving rise to $CD146^{+}$ stromal

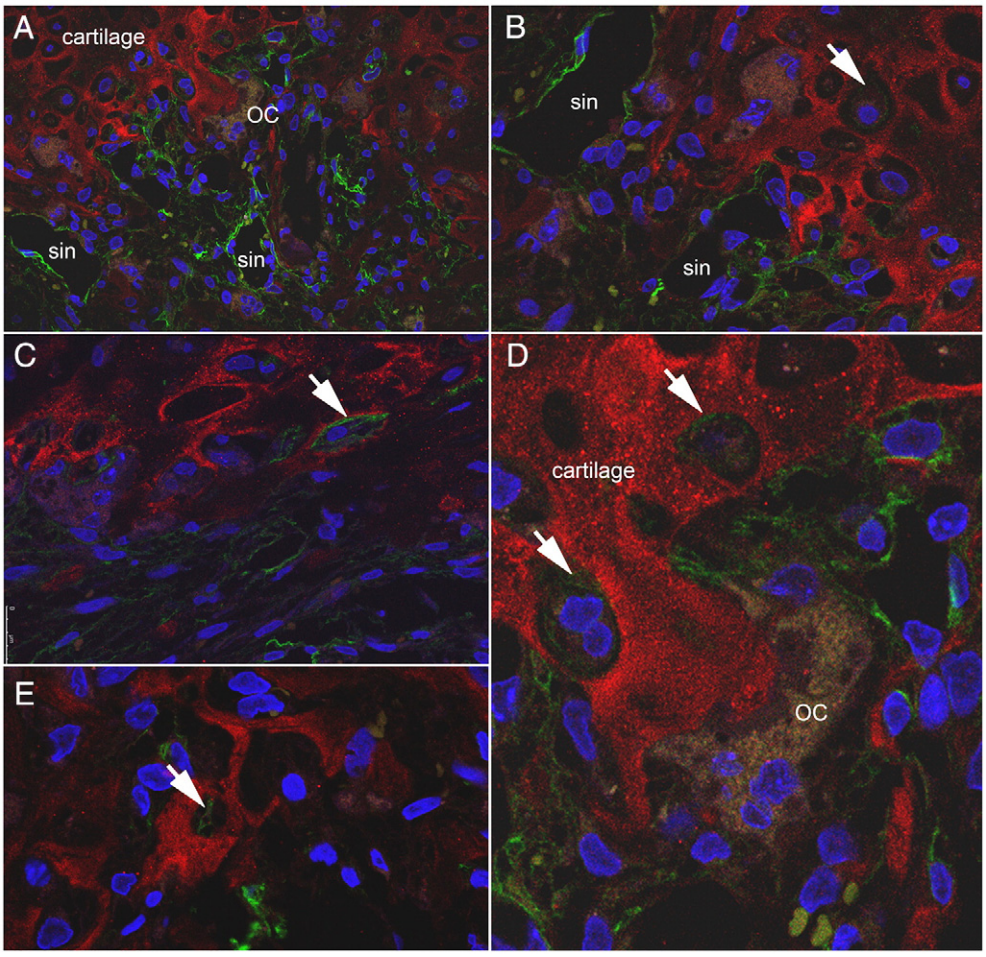


Figure 5 Remodeling of pre-existing cartilage into marrow stroma in vivo. Confocal fluorescence images of the resorbing cartilage/growing marrow interface. Col2 immunoreactive cartilage is shown in red; hCD146-expressing cells in green. Osteoclasts (oc), visualized by autofluorescence, resorb cartilage matrix and open up chondrocyte lacunae. Elongated, branched stromal cells expressing hCD146 populate the newly opened lacunae (A, D). Individual chondrocytes within lacunae near the resorption front express low levels of hCD146 (arrows in B–E). Maximum intensity of CD146 signal is seen in cells covering sinusoid-like blood vessels (sin).

progenies upon growth on plastic. Taken together, these data demonstrated that chondroid differentiation of BMSC cultures, as achieved per standard protocols of ex vivo "differentiation," in fact represents a completely reversible modulation of cartilage and stromal phenotypes in opposite directions.

Chondrocyte-derived stromal cells are functional skeletal stem cells

We then asked if the CD146$^+$ stromal cells populating the heterotopic ossicles in vivo, exhibited functional properties of bone marrow stromal stem cells (bone marrow-derived "MSCs"). To this end, the ossicles that formed following transplantation of the cartilage pellets were harvested at 8 weeks and collagenase digested to obtain single cell suspensions. The human CD146$^+$ and CD146$^-$ fractions were then sorted and plated in culture. Colony forming efficiency assays demonstrated that CFU-Fs were segregated and highly frequent (20%) into the hCD146$^+$ fraction (Figs. 7A, B), and generated clonal colonies that expressed CD146 uniformly at high levels (Fig. 7C). All "canonical" markers of "MSCs" (Dominici et al., 2006) were highly expressed in cultures of human CD146$^+$ cells from the heterotopic ossicles generated by transplanted pellets (Fig. 7D). Secondary cartilage pellets from pooled colonies were readily generated from such cultures upon exposure to chondrogenic conditions (Figs. 7E,F). Secondary transplantation of the secondary pellets again resulted in heterotopic ossicles (Fig. 7G), indicating that stromal cells emerging from chondroid pellets in vivo, and recruited to an adventitial perisinusoidal position in the heterotopic marrow retained clonogenicity, self-renewal capacity, and the ability to differentiate into cartilage and bone in vivo.

Discussion

"Differentiation" of stromal cells into chondrocytes is a widely assumed property of stromal cells (in particular, for BMSCs), and is canonically assayed in pellet cultures (Johnstone et al., 1998; Muraglia et al., 2003; Sekiya et al., 2002). We have shown that in vitro and in vivo, BMSC populations can be induced to fluctuate between a stromal and a cartilage

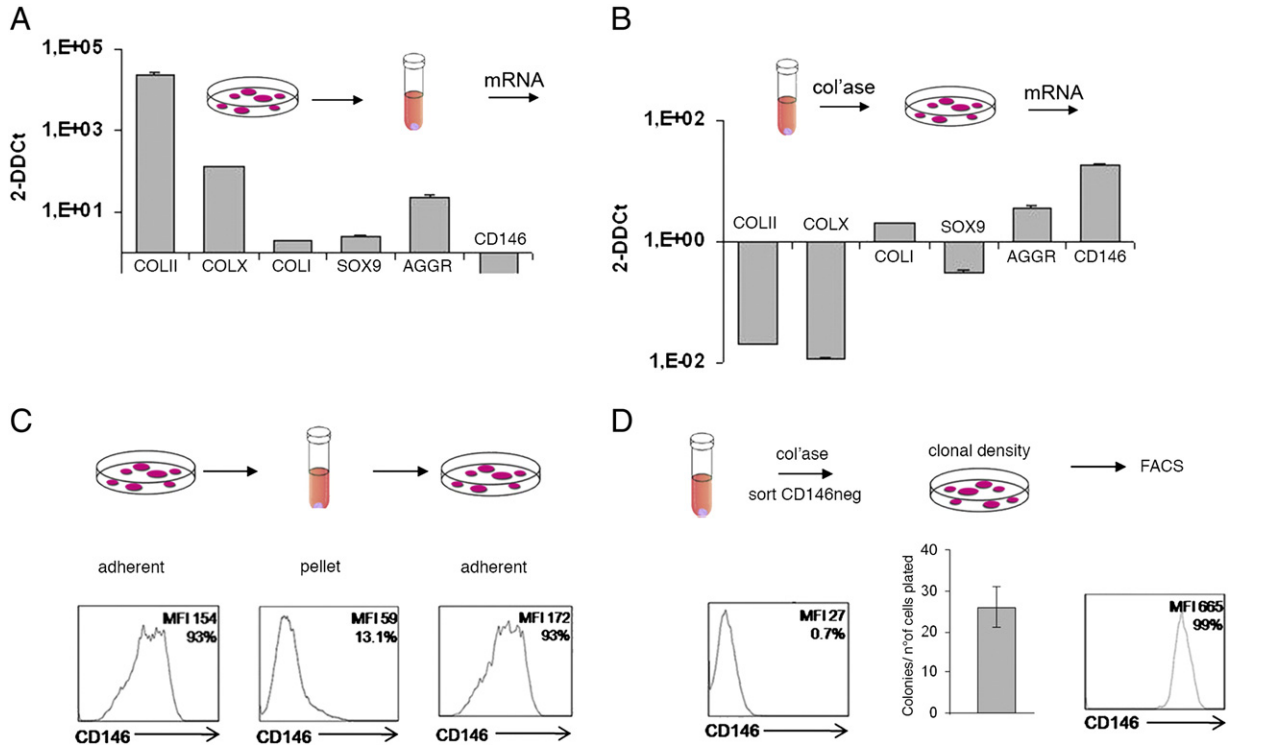
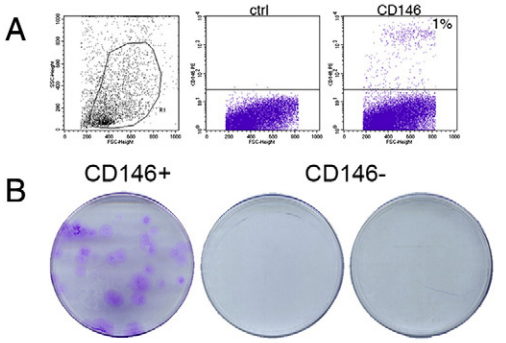


Figure 6 Reversible modulation of cartilage and stromal phenotypes in vitro. A) Modulation of stromal and cartilage gene expression upon induction of chondroid differentiation in primary cultures of BMSCs. q-RT-PCR. B) Reverse modulation of expression of the same genes upon establishment of secondary cultures of BMSCs from collagenase released pellet cells of cartilage. q-RT-PCR. C) Modulation of expression of CD146 protein. FACS analyses of the same population are shown in primary adherent culture (left), following collagenase release from pellets (middle), and after secondary adherent culture (right). CD146 expression is modulated in opposite direction when adherent cells are transferred to micromass (non-adherent) culture, or when micromass cultured cells are replated in adherent culture. Low residual levels of CD146 expression are detected in micromass cultured (pellet) cells. Secondary cultures could be induced to secondary pellet formation. D) Induction of CD146 expression in $CD146^-$ pellet cells following collagenase release, sorting and plating as adherent cells at clonal density. FACS analysis of $CD146^-$ sorted cells prior to plating in culture, and at 14 days of secondary culture. Colonies were pooled for FACS analysis.

phenotype. We have shown that secondary pellets can be generated from cells released as $CD146^-$ chondrocytes from the primary pellet, and secondarily passaged as $CD146^+$ stroma. We have also shown that stromal cells can indeed self-renew while traversing a complex sequence of events that can include not only serial transplantation, but also multiple ex vivo growth and differentiation steps. This implies that "differentiation" of bone marrow stromal cells into chondrocytes in vitro is a reversible event. It remains to be determined if: a) such reversibility is a culture epiphenomenon, in which case the pellet culture system would not, in fact, be mirroring the physiological chondrogenic differentiation, or b) it reflects the behavior of chondrocytes existing in natural cartilage (at least at specific times and sites), in which case, cartilage itself would come to be seen as a reservoir of reversibly differentiated cells. Multiple evidences for the fact that cartilage may not necessarily represent terminal differentiation have been previously given. For example, chick embryonic chondrocytes can revert to fibroblast-like cells if plated as adherent cells; these adherent cells can then be induced to hypertrophic chondrocytes in suspension cultures, and an osteoblast-like phenotype can be further induced in chondrocytes replated as adherent cells (Castagnola et al., 1988; Castagnola et al., 1986; Galotto et al., 1994; Gentili et al., 1993; Tacchetti et al., 1987). Chondrocytes in "metamorphic" cartilages (Holmbeck et al., 2003) in the mouse (such as parietal cartilage or Meckel's cartilage), can activate MT1-MMP and use it to degrade and escape from unmineralized cartilage (Holmbeck et al., 2003). Mammalian chondrocytes located next to the bony collar in developing bones can express a host of osteogenic markers (Bianco et al., 1998; Riminucci et al., 1998). Instability of the "chondrocyte" phenotype in pellets obtained from "MSCs" had been previously noted (Dickhut et al., 2009; Pelttari et al., 2006). In addition, dedifferentiation of chondrocytes in cartilaginous tumors is a common event and a popular concept in human pathology (Dorfman and Czerniak, 1998). We have shown here that the chondrocytic phenotype induced by pellet culture of human BMSCs is reversible in vitro and in vivo. This reversibility can account for the origin of the marrow stromal cells that populate the pellet-generated bone marrow in vivo in our transplantation system that is free of exogenous scaffolds. A remarkable degree of reversibility thus denotes the cartilage differentiation of human BMSCs as canonically assessed by in vitro assays. As we have shown, a chondrocyte phenotype can be induced and then reversed, and then induced back again. Stromal cells induced to become chondrocytes



CFE assay for CD146⁺ and CD146⁻ ossicle-derived cells

Cells plated	*Cell density (cells/cm²)*	*CFU-F*	
		CD146⁺	CD146⁻
1x10²	1.6	25.7 + 6	0
1x10³	16	ND	0
3x10⁵	5x10³	ND	confluency

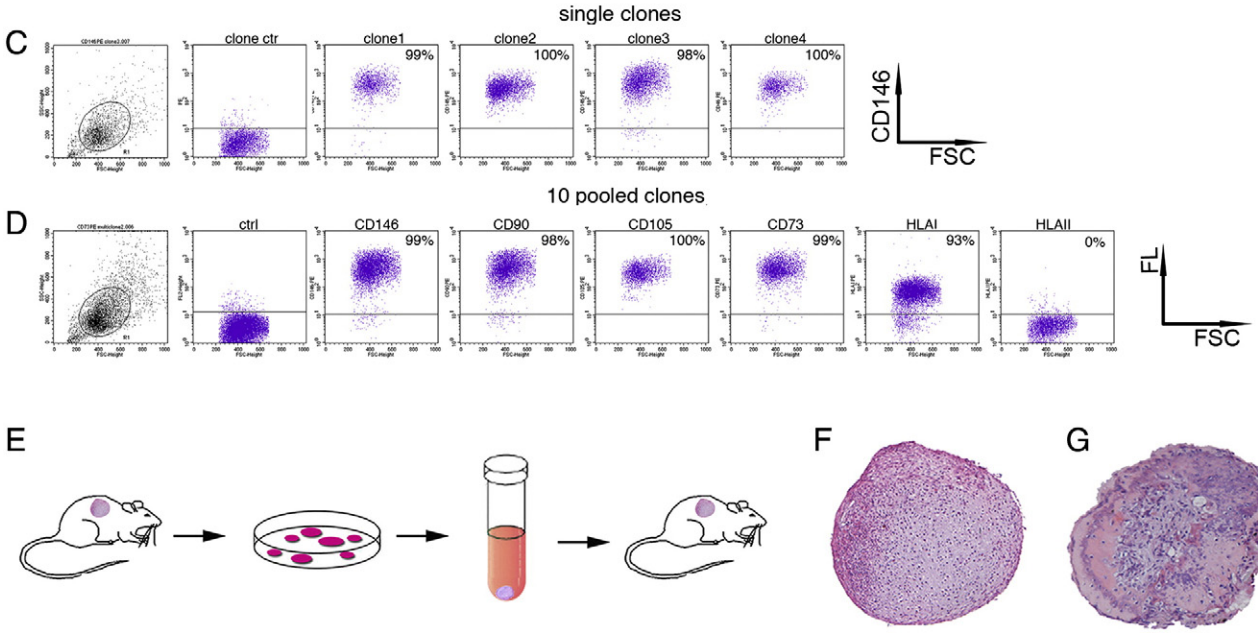


Figure 7 Stem cell properties of human stromal cells isolated from heterotopic ossicles. A) Expression of hCD146 in collagenase released cells from heterotopic ossicles (8 weeks). B) CFE assays of sorted CD146⁻ and CD146⁻ fractions, revealing restriction of hCFU-Fs to the positive fraction, and high clonogenic efficiency. C) Uniform expression of CD146 in 4 single clones of stromal cells in secondary culture. D) Phenotype of pooled colonies of stromal cells in secondary culture, demonstrating uniform expression of CD146 and of canonical markers of "MSCs." E) Secondary in vitro differentiation and in vivo transplantation of secondarily cultured stromal cells. F) chondroid pellet; G) secondary ossicle.

can in turn revert back to stromal cells, while retaining their functional potential. In particular, they can revert to hematopoiesis-supporting cells, establish a hematopoietic stem cell niche, and resume properties characteristic of self-renewing skeletal ("mesenchymal") stem cells.

Ossicles generated from pellets reproduce the architecture of natural bones (cortical bone, medullary cavity), which therefore can definitely be established independent of any instructive cue provided by any exogenous scaffolds. Positioning bone and the marrow cavity in the precise spatial pattern that mimics, in an artificial spheroid structure, the natural layout must then depend on self-organization, and reflect cues that operate either in the pre-transplantation pellet or in the transplant in vivo. Earlier work had shown that in cartilage pellets made from BMSCs, an outer layer of cells expressing bone matrix proteins differentiates at the periphery of the pellet and deposits a mineralized phase upon subculturing under mineralizing conditions (Muraglia et al., 2003). This suggests that the human bone that forms at the periphery of the transplanted, unmineralized pellets is in fact the product of cells that acquire the competence to form bone prior to transplantation, and specifically reside at the pellet periphery. Multiple determinants can be envisioned to contribute to the establishment of differential milieus in the peripheral and central portions of the pellet, respectively, such as an outside-in oxygen gradient, or surface tension. The general architectural plan of the ossicle (position of the presumptive marrow "niche" and bony cortex) generated by the pellet transplant may thus be pre-defined in the ex vivo pellet by these particular cues.

In our system, cartilage is replaced directly by marrow, not by bone. A spatially ordered sequence of chondrocyte proliferation, hypertrophy, and apoptosis is not reproduced in the developing pellet-derived ossicles. Thus, the establishment

of heterotopic bone and bone marrow in our system is not dependent on mimicry of physiological endochondral ossification. It depends on a different process, which involves the removal of cartilage by chondroclasts/osteoclasts and its replacement by marrow. The local hematopoietic microenvironment/"niche" is established by stromal cells, which are locally formed. Such stromal cells in our system may originate in vivo from chondrocytes within the pellet, which survive, turn on expression of the stromal marker, CD146, and can physically escape from cartilage once their lacuna is opened up by chondroclasts/osteoclasts.

Our data point to reversibility as a neglected dimension of the categories "differentiation potential," and "stemness" as commonly applied to so-called "mesenchymal stem cells." In this system of non-epithelial, mesoderm-derived tissues [the "stromal system" of Friedenstein and Owen (Owen, 1988)], phenotypic instability and modulation of phenotype within the range of skeletal tissue types may be unique among stem cell-ruled systems.

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References

Adachi, N., Ochi, M., Deie, M., Ito, Y., 2005. Transplant of mesenchymal stem cells and hydroxyapatite ceramics to treat severe osteochondral damage after septic arthritis of the knee. J. Rheumatol. 32, 1615–1618.

Adolfsson, J., Borge, O.J., Bryder, D., Theilgaard-Monch, K., Astrand-Grundstrom, I., Sitnicka, E., Sasaki, Y., Jacobsen, S.E., 2001. Upregulation of Flt3 expression within the bone marrow Lin(−)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. Immunity 15, 659–669.

Akashi, K., Traver, D., Miyamoto, T., Weissman, I.L., 2000. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature 404, 193–197.

Bianco, P., 2011. Bone and the hematopoietic niche: a tale of two stem cells. Blood 117, 5281–5288.

Bianco, P., Ponzi, A., Bonucci, E., 1984. Basic and 'special' stains for plastic sections in bone marrow histopathology, with special reference to May-Grunwald Giemsa and enzyme histochemistry. Basic Appl. Histochem. 28, 265–279.

Bianco, P., Cancedda, F.D., Riminucci, M., Cancedda, R., 1998. Bone formation via cartilage models: the "borderline" chondrocyte. Matrix Biol. 17, 185–192.

Bianco, P., Cao, X., Frenette, P.S., Mao, J.J., Robey, P.G., Simmons, P.J., Wang, C.Y., 2013. The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. Nat. Med. 19, 35–42.

Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R., et al., 2003. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 425, 841–846.

Castagnola, P., Moro, G., Descalzi-Cancedda, F., Cancedda, R., 1986. Type X collagen synthesis during in vitro development of chick embryo tibial chondrocytes. J. Cell Biol. 102, 2310–2317.

Castagnola, P., Dozin, B., Moro, G., Cancedda, R., 1988. Changes in the expression of collagen genes show two stages in chondrocyte differentiation in vitro. J. Cell Biol. 106, 461–467.

Dickhut, A., Pelttari, K., Janicki, P., Wagner, W., Eckstein, V., Egermann, M., Richter, W., 2009. Calcification or dedifferentiation: requirement to lock mesenchymal stem cells in a desired differentiation stage. J. Cell. Physiol. 219, 219–226.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., Horwitz, E., 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8, 315–317.

Dorfman, H.D., Czerniak, B., 1998. Bone Tumors. Mosby, St Louis.

Friedenstein, A.J., 1990. Osteogenic stem cells in bone marrow. In: Heersche, J.N.M., Kanis, J.A. (Eds.), In Bone and Mineral Research. Elsevier, Amsterdam, pp. 243–272.

Friedenstein, A.J., Chailakhjan, R.K., Lalykina, K.S., 1970. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet. 3, 393–403.

Galotto, M., Campanile, G., Robino, G., Cancedda, F.D., Bianco, P., Cancedda, R., 1994. Hypertrophic chondrocytes undergo further differentiation to osteoblast-like cells and participate in the initial bone formation in developing chick embryo. J. Bone Miner. Res. 9, 1239–1249.

Gentili, C., Bianco, P., Neri, M., Malpeli, M., Campanile, G., Castagnola, P., Cancedda, R., Cancedda, F.D., 1993. Cell proliferation, extracellular matrix mineralization, and ovotransferrin transient expression during in vitro differentiation of chick hypertrophic chondrocytes into osteoblast-like cells. J. Cell Biol. 122, 703–712.

Holmbeck, K., Bianco, P., Caterina, J., Yamada, S., Kromer, M., Kuznetsov, S.A., Mankani, M., Robey, P.G., Poole, A.R., Pidoux, I., et al., 1999. MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. Cell 99, 81–92.

Holmbeck, K., Bianco, P., Chrysovergis, K., Yamada, S., Birkedal-Hansen, H., 2003. MT1-MMP-dependent, apoptotic remodeling of unmineralized cartilage: a critical process in skeletal growth. J. Cell Biol. 163, 661–671.

Johnstone, B., Hering, T.M., Caplan, A.I., Goldberg, V.M., Yoo, J.U., 1998. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp. Cell Res. 238, 265–272.

Krebsbach, P.H., Kuznetsov, S.A., Satomura, K., Emmons, R.V., Rowe, D.W., Robey, P.G., 1997. Bone formation in vivo: comparison of osteogenesis by transplanted mouse and human marrow stromal fibroblasts. Transplantation 63, 1059–1069.

Kronenberg, H.M., 2003. Developmental regulation of the growth plate. Nature 423, 332–336.

Kuznetsov, S.A., Krebsbach, P.H., Satomura, K., Kerr, J., Riminucci, M., Benayahu, D., Robey, P.G., 1997. Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. J. Bone Miner. Res. 12, 1335–1347.

Mankani, M.H., Kuznetsov, S.A., Fowler, B., Kingman, A., Robey, P.G., 2001. In vivo bone formation by human bone marrow stromal cells: effect of carrier particle size and shape. Biotechnol. Bioeng. 72, 96–107.

Mankani, M.H., Kuznetsov, S.A., Marshall, G.W., Robey, P.G., 2008. Creation of new bone by the percutaneous injection of human bone marrow stromal cell and HA/TCP suspensions. Tissue Eng. Part A 14, 1949–1958.

Mendez-Ferrer, S., Michurina, T.V., Ferraro, F., Mazloom, A.R., Macarthur, B.D., Lira, S.A., Scadden, D.T., Ma'ayan, A., Enikolopov, G.N., Frenette, P.S., 2010. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature 466, 829–834.

Muraglia, A., Corsi, A., Riminucci, M., Mastrogiacomo, M., Cancedda, R., Bianco, P., Quarto, R., 2003. Formation of a chondro-osseous rudiment in micromass cultures of human bone-marrow stromal cells. J. Cell Sci. 116, 2949–2955.

Owen, M., 1988. Marrow stromal stem cells. J. Cell Sci. Suppl. 10, 63–76.

Pelttari, K., Winter, A., Steck, E., Goetzke, K., Hennig, T., Ochs, B.G., Aigner, T., Richter, W., 2006. Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. Arthritis Rheum. 54, 3254–3266.

Quarto, R., Mastrogiacomo, M., Cancedda, R., Kutepov, S.M., Mukhachev, V., Lavroukov, A., Kon, E., Marcacci, M., 2001. Repair of large bone defects with the use of autologous bone marrow stromal cells. N. Engl. J. Med. 344, 385–386.

Riminucci, M., Bradbeer, J.N., Corsi, A., Gentili, C., Descalzi, F., Cancedda, R., Bianco, P., 1998. Vis-a-vis cells and the priming of bone formation. J. Bone Miner. Res. 13, 1852–1861.

Robertson, D., Savage, K., Reis-Filho, J.S., Isacke, C.M., 2008. Multiple immunofluorescence labelling of formalin-fixed paraffin-embedded (FFPE) tissue. BMC Cell Biol. 9, 13.

Sacchetti, B., Funari, A., Michienzi, S., Di Cesare, S., Piersanti, S., Saggio, I., Tagliafico, E., Ferrari, S., Robey, P.G., Riminucci, M., et al., 2007. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell 131, 324–336.

Sekiya, I., Vuoristo, J.T., Larson, B.L., Prockop, D.J., 2002. In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. Proc. Natl. Acad. Sci. U. S. A. 99, 4397–4402.

Tacchetti, C., Quarto, R., Nitsch, L., Hartmann, D.J., Cancedda, R., 1987. In vitro morphogenesis of chick embryo hypertrophic cartilage. J. Cell Biol. 105, 999–1006.

Tavassoli, M., Crosby, W.H., 1968. Transplantation of marrow to extramedullary sites. Science 161, 54–56.