INTRODUCTION

Bone-derived mesenchymal stromal cells (MSCs) differentiate into multiple lineages including chondro- and osteogenic fates and function in establishing the hematopoietic compartment of the bone marrow. Here, we analyze the emergence of different MSC types during mouse limb and long bone development. In particular, PDGFRα⁺SCA-1⁺ (PαS) cells and mouse skeletal stem cells (mSSCs) are detected within the PDGFRα⁺CD51⁺ (PaCD51) mesenchymal progenitors, which are the most abundant progenitors in early limb buds and developing long bones until birth. Long-bone-derived PαS cells and mSSCs are most prevalent in newborn mice, and molecular analysis shows that they constitute distinct progenitor populations from the earliest stages onward. Differential expression of CD90 and CD73 identifies four PαS subpopulations that display distinct chondro- and osteogenic differentiation potentials. Finally, we show that cartilage constructs generated from CD90⁺ PαS cells are remodeled into bone organoids encompassing functional endothelial and hematopoietic compartments, which makes these cells suited for bone tissue engineering.

SUMMARY

A wealth of studies using bone-derived mesenchymal stromal cells (MSCs) has revealed their importance for engineering to repair cartilage and bone tissues, and for ameliorating hematopoietic disorders (Bianco, 2014). However, it is still not firmly established whether MSC populations encompass mesenchymal progenitor and/or stem cells. In a recent review, Caplan (2017) proposes that MSCs, rather than being progenitor/stem cells, home to sites of injury and secrete factors that induce regeneration by resident stem cells. As it is indeed difficult to expand MSCs in culture without affecting their initial characteristics (Bianco, 2014; Mabuchi et al., 2013), the prospective isolation and direct analysis of primary mesenchymal progenitors from human bone marrow and mouse long bones is central to identify their origins and assessing their multi-lineage differentiation potential. Since the first isolation of clonogenic mesenchymal progenitors from human bone marrow (Sacchetti et al., 2007), lineage tracing and prospective characterization using mouse models has identified different types of mesenchymal progenitor/stem cells and provided fundamental insights into their functions during normal bone homeostasis and repair (Morrison and Scadden, 2014; Ono and Kronenberg, 2016). However, the relatedness of these different mesenchymal populations from mice and their corresponding human orthologs remains uncertain.

Studying mouse limb development provides insight into the ontology and functions of the mesenchymal progenitors that orchestrate development of long bones (reviewed by Long and Ornitz, 2013). In brief, the development of the skeletal primordia in early mouse limb buds is initiated by the condensation of multipotent mesenchymal progenitors at around embryonic day 10.5 (E10.5) and their commitment as Sox9-positive osteo-chondrogenic progenitors (Akiyama et al., 2005), which will form the cartilage primordia. The SOX9-positive cells differentiate into proliferating chondrocytes while peripheral mesenchymal cells will form the perichondrium (Akiyama and Lefebvre, 2011). Endochondral ossification is initiated after chondrocytes become hypertrophic (~E12.5), which involves differentiation of perichondrium progenitors into Osterix (Osx) expressing osteoblastic and mesenchymal progenitors (Liu et al., 2013; Maes et al., 2010; Mizoguchi et al., 2014; Ono et al., 2014a). Limb long bone growth and bone marrow formation depends on angiogenesis, which is triggered by vascular endothelial growth factor (VEGF)-mediated attraction of endothelial and hematopoietic progenitors (≥E14.5; Morrison and Scadden, 2014). The main migration of hematopoietic stem cells (HSCs) from the fetal liver to the bone marrow initiates perinatally, peaks immediately after birth, and continues until puberty, after which bone homeostasis is achieved (Kim et al., 2007; Trumpp et al., 2010). Concurrently, the cellular composition of the mesenchymal stromal compartment changes to support hematopoiesis (Greenbaum et al., 2013; Maes et al.,...
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Recently, CD200posCD51pos mesenchymal progenitors (lack± results are presented as averages experiments (Ono et al., 2014b; Park et al., 2012). More Col2a populations marked in 2012; Hu et al., 2016; Morikawa et al., 2009; Park et al., 2013). P hindlimb long bones at all subsequent stages. Red cell lysis was included for older stages (W4; juvenile stage), and W10–13 (adult stages) were analyzed by flow cytometry. The analysis was done from forelimb buds at E10.5 and (A–D) Cells isolated from total limb buds at embryonic day 10.5 (E10.5), long bones at E14.5–15.5, E18.5, postnatal days 1–3 (P1–3), week 4 (W4; juvenile stage), and W10–13 (adult stages) were analyzed by flow cytometry. The analysis was done from forelimb buds at E10.5 and hindlimb long bones at all subsequent stages. Red cell lysis was included for older stages (≥W4). The lineage marker pool (Lin) includes the following antigens: CD45, TER119, CD31, Gr1, and CD11b (all stages) plus EpCAM and CD309 (only for limb buds). Dead cells were gated out using 7AAD. Results are shown as pseudo-color plot representations of the subsequent analysis of the lineage-negative (Linneg) fraction. (A and B) Distribution of Linneg cells expressing either the platelet-derived growth factor receptor α (PDGFRα)/CD51 or PDGFRα/SCA-1 antigens. Note that in limb buds at E10.5, CD51 is assessed against SCA-1 (B, see text). (C) Overlapping dot plot representations show that PzS cells (blue) are mostly contained in the PzCD51 population (gray). (D) Fraction of the SCA-1-positive PzCD51 cells corresponding to PzS cells. (E) Percentage of PzCD51- and Pz-positive cells in the Linneg population. Per stage n ≥ 3 independent experiments were analyzed. All results are presented as averages ± SD.

Figure 1. Ontogenic Identification of Mesenchymal Stromal Cells during Embryonic, Fetal, and Postnatal Limb Bud and Long Bone Development

(A–D) Cells isolated from total limb buds at embryonic day 10.5 (E10.5), long bones at E14.5–15.5, E18.5, postnatal days 1–3 (P1–3), week 4 (W4; juvenile stage), and W10–13 (adult stages) were analyzed by flow cytometry. The analysis was done from forelimb buds at E10.5 and hindlimb long bones at all subsequent stages. Red cell lysis was included for older stages (≥W4). The lineage marker pool (Lin) includes the following antigens: CD45, TER119, CD31, Gr1, and CD11b (all stages) plus EpCAM and CD309 (only for limb buds). Dead cells were gated out using 7AAD. Results are shown as pseudo-color plot representations of the subsequent analysis of the lineage-negative (Linneg) fraction. (A and B) Distribution of Linneg cells expressing either the platelet-derived growth factor receptor α (PDGFRα)/CD51 or PDGFRα/SCA-1 antigens. Note that in limb buds at E10.5, CD51 is assessed against SCA-1 (B, see text). (C) Overlapping dot plot representations show that PzS cells (blue) are mostly contained in the PzCD51 population (gray). (D) Fraction of the SCA-1-positive PzCD51 cells corresponding to PzS cells. (E) Percentage of PzCD51- and Pz-positive cells in the Linneg population. Per stage n ≥ 3 independent experiments were analyzed. All results are presented as averages ± SD.
identify mesenchymal cells with signatures of different MSC populations. Following preparation of single-cell suspensions, dead cells, endothelial, hematopoietic, and epithelial/ectodermal cells were first removed using the appropriate cell-surface markers (see Supplemental Experimental Procedures). An ontogenetic flow-cytometric analysis was done using the remaining so-called lineage-negative (Lin\textsuperscript{neg}) cells from the different stages (Figures 1 and 2). Initially, the signatures of two types of MSC, namely P\textalpha{}cCD51 and P\textalpha{}c progenitors, were profiled (Figure 1). In developing mouse limb buds (E10.5 and E14.5–15.5), the vast majority of Lin\textsuperscript{neg} mesenchymal progenitors are P\textalpha{}cCD51 positive (~80%–95%, Figures 1A and 1E). During fetal long bone development (E14.5–18.5), P\textalpha{}cCD51-positive cells (also including osteoblasts; Chitteti et al., 2013) remain most prominent (Figure 1E, purple bars). During early postnatal development (P1–3), P\textalpha{}cCD51 cells account for ~50% of the Lin\textsuperscript{neg} cells, but their frequency drops to ~4% in juvenile and adult long bones. This decrease in P\textalpha{}cCD51 cells is paralleled by a significant increase in PDGFR\textalpha{} single-positive (P\textalpha{}) cells after birth (Figures 1A and 1E).

In contrast to the predominant P\textalpha{}cCD51 population, P\textalpha{} cells are less abundant (Figure 1B). In early limb buds (E10.5), SCA-1-positive mesenchymal progenitors express CD51 and intermediate levels of PDGFR\alpha{} (Figures 1B–1D). From E14.5 onward, the P\textalpha{} cell population increases progressively (Figure 1C) such that the highest proportion is observed around birth (~30% at E18.5 and P1–3, Figure 1B). This analysis (Figure 1) establishes that the P\textalpha{}cCD51-positive cells encompass the P\textalpha{} cell population at all stages. This is relevant to potential therapeutic applications as, in contrast to CD51, the SCA-1 antigen is not present in humans (Lee et al., 2013).

P\textalpha{} and mSSC Signatures Identify Two Distinct Progenitor Populations within the P\textalpha{}cCD51 Mesenchymal Cell Pool

We next analyzed the relationship between mSSC (CD51\textsuperscript{pos} CD200\textsuperscript{pos} CD90\textsuperscript{neg}CD105\textsuperscript{neg} 6C3\textsuperscript{neg} cells; Chan et al., 2015), P\textalpha{}cCD51, and P\textalpha{} populations (Figure 2). Within the Lin\textsuperscript{neg} cells, CD51 is key to defining the predominant P\textalpha{}cCD51 population and SCA-1 identifies the P\textalpha{} cells within this population (Figure 2A). In addition, the distribution of SCA-1- and CD200-positive cells within Lin\textsuperscript{neg}CD51\textsuperscript{pos} cell pool was determined (Figure 2B). Strikingly, this analysis establishes that the SCA-1\textsuperscript{pos} P\textalpha{} cells and CD200\textsuperscript{pos} mSSCs segregate as mutually exclusive populations at all stages (Figure 2B). In early mouse limb buds (E10.5), the CD51\textsuperscript{pos}SCA-1\textsuperscript{pos} P\textalpha{} cells are much more abundant than CD51\textsuperscript{pos}CD200\textsuperscript{pos} cells (5% versus 0.5%; Figures 2A, 2B, and 2E). During fetal and early postnatal long bone development (E14.5–P2), P\textalpha{} cells prevail over mSSCs (Figure 2E). During peak bone angiogenesis and invasion of HSCs into long bones immediately after birth (Trumpp et al., 2010), P\textalpha{} cells account for about half of all P\textalpha{}cCD51 cells. During puberty around week 4 (W4), mSSCs are about twice as frequent as P\textalpha{} cells, while these populations are similarly represented in adult long bones (Figures 2B and 2E).

To gain insight into the potential overlap of these two cell populations with Sox9-positive progenitors, we included the Sox9\textsuperscript{IRES-GFP} transgene in our analysis (Chan et al., 2011). In early limb buds (E10.5), the Sox9-GFP-positive cells correspond mostly to osteo-chondroprogenitors (Akiyama et al., 2005 and our unpublished data). During subsequent development, Sox9 is expressed by chondroblasts, reduced in proliferating chondrocytes, and re-expressed in pre-hypertrophic chondrocytes (Akiyama et al., 2002; Dy et al., 2012). While Sox9-GFP levels are always low in P\textalpha{} cells, the majority of mSSCs express intermediate to high levels of Sox9 (Figures 2C and 2D).

As the Prx1-Cre transgene is expressed by most limb bud mesenchymal progenitors, we used it to activate a conditional GFP reporter (\(\beta\text{-ACTIN-loxP-stop-loxP-EGFP}\); Jagle et al., 2007; Logan et al., 2002). This allowed us to determine the fractions of P\textalpha{}cCD51, P\textalpha{}, and mSSC populations in GFP-positive cells at P2 (Figure S1). This analysis revealed that all GFP-positive cells are contained in the P\textalpha{}cCD51 population, which in turn shows that all P\textalpha{} cells and mSSCs are GFP positive, i.e., are either Prx1-expressing cells or their descendants. In contrast the P\textalpha{} cells, which become predominant during postnatal development, are GFP negative (Figure S1). This analysis establishes P\textalpha{}cCD51-positive cells as the major cell population within the mesenchymal (stromal) compartment during embryonic limb bud and fetal long bone development.

Next, the different mesenchymal populations in long bones after birth (P1–3) and during adult bone homeostasis (W10–13) (Figure 3) were analyzed for the expression of key genes relevant to chondrogenesis, osteogenesis, and hematopoiesis (Prx1, Col2a1, and Mmp13; Osx, Lepr, and Cxcl12), respectively. qRT-PCR analysis of the different mesenchymal populations (Figure 3A) showed that the different cell populations express distinct levels of these genes in a rather consistent manner when comparing newborn and adult mice. The most prominent Lin\textsuperscript{neg} population in adults, P\textalpha{} cells (Figure 1B), express none of these genes at birth and very variable levels of Lepr in adults (Figures 3B and 3C). Therefore, P\textalpha{} cells are unlikely to possess robust chondrogenic and osteogenic differentiation potential. P\textalpha{}cCD51 cells, P\textalpha{} cells, and mSSCs express Prx1 at birth and in adults. While P\textalpha{} cells do not express chondrogenic and osteogenic markers, they express the highest levels of Prx1 and low to intermediate levels of Cxcl12 and Lepr at both stages (Figures 3B and 3C). This expression pattern
Figure 2. Ontogenic Analysis of Cells with mSSC and PαS Signatures

Flow-cytometric identification of different mesenchymal cell types in the Lin<sup>neg</sup> fraction isolated from limb buds (E10.5–14.5) and limb long bones (E18.5–W12) of Sox9-GFP mice.

(A) Pseudo-color plot distribution of CD51- and SCA-1 positive Lin<sup>neg</sup> cells. Note that the PαS cells are almost completely contained within the CD51<sup>pos</sup>SCA-1<sup>pos</sup> cell population.

(B) Analysis of CD51<sup>pos</sup> Lin<sup>neg</sup> cells with CD200 and SCA-1 shows that the CD200<sup>pos</sup> (including mSSC) and SCA-1<sup>pos</sup> cells (including PαS) define distinct cell populations at all stages.

(C) Additional flow-cytometric analysis identifies mSSCs as CD90<sup>neg</sup>CD105<sup>neg</sup>6C3<sup>neg</sup> subpopulation within the CD51<sup>pos</sup>CD200<sup>pos</sup> fraction (percentage indicated within the yellow-green frame). Note that a significant fraction of mSSCs expresses Sox9-GFP from E13.5 and in particular from W4 onward.

(D) Variable expression of Sox9-GFP and the CD90, CD105, and 6C3 antigens in PαS cells.

(E) Graph showing the percentage of PαS cells and mSSCs in the Lin<sup>neg</sup>CD51<sup>pos</sup> population. Per time point and cell type n ≥ 3 independent experiments were analyzed. All results are presented as averages ± SD. Unpaired 2-tailed Student’s t test was used to assess significance.

* p < 0.05, ** p < 0.01, *** p < 0.001 were considered statistically significant.
points to the likely immature nature of PαS cells and is in agreement with their potential to support HSCs (see below). mSSCs isolated from adult long bones express high levels of Col2a1, Mmp13, and, notably, Osx (Figure 3C), which corroborates their osteo-chondrogenic potential (Chan et al., 2015). Finally, SCA-1negCD200neg PαCD51DN (PαCD51DN) cells express the highest levels of Cxcl12 and Lepr while all other genes are expressed at only low to intermediate levels. These results indicate that the PαCD51DN population could encompass CAR cells (CXCL12-abundant reticular cells), which support B cell development (Greenbaum et al., 2013; Omatsu et al., 2010; Sugiyama et al., 2006). Taken together, this analysis of few selected key genes corroborates the distinct nature
of the different mesenchymal stromal progenitor populations identified by flow cytometry.

**PαS Cells Are a Heterogeneous Population Consisting of Subpopulations with Distinct CD Signatures**

The PαS population was analyzed in more detail, as these cells are detected from early limb bud stages onward (see above). We performed the same analysis as shown in Figures 1 and 2 but also assessing the expression of CD73 and CD90 (Figure 4). CD73 is expressed by chondrocyte progenitors and CD90 marks the osteogenic lineage (see Introduction). Analysis of these two markers using freshly isolated adult PαS cells identifies four distinct subpopulations either negative or positive for CD90 and expressing intermediate (im) or high (hi) levels of CD73 (CD90negCD73im, CD90negCD73hi, and CD90posCD73im, CD90posCD73hi; Figure 4A). In vitro expansion of PαS cells causes a shift in the abundance of the four subpopulations. After two passages, the fractions of the two CD90neg subpopulations are much reduced, while the CD90pos PαS subpopulations become predominant (Figure 4B). This reduction is paralleled by a decrease in the multipotency, which affects mostly the osteo-chondrogenic differentiation potential (data not shown).

To understand when these four PαS subpopulations arise during limb long bone development, we analyzed Linneg cells isolated from Sox9-GFP expressing embryos and mice by flow cytometry (Figure 5). During embryonic, fetal, and early postnatal stages, the Sox9-GFP-positive cells that mark the osteo-chondrogenic lineage (Figure 5A) comprise a significant fraction of all Linneg mesenchymal (stromal) cells (Figure 5E). From about 2 weeks postnatally, the fraction of Sox9-GFP cells within the Linneg fraction decreases significantly (Figures 5A and 5E). Concurrently, there is a switch in the proportions of the CD90pos and CD90neg PαS subpopulations (Figures 5E and 5F).

Mesenchymal SCA-1pos cells in early limb buds express CD51 and intermediate levels of PDGFRα (E10.5; Figure 5B, compare with Figures 1 and 2). At this stage, Sca-1 is expressed by the undifferentiated mesenchymal progenitors located distally and close to the limb bud ectoderm (Figure S2A; ten Berge et al., 2008). Furthermore, PαS cells express intermediate levels of CD73 (CD73im) while CD90 is not detected (E10.5; Figures 5B, 5C, and S2B). The PαS progenitors are a heterogeneous population, as 10%–15% of them are also Sox9-GFP positive in early limb buds (Figure 5D). In fact, the majority of Sox9-GFP-positive progenitors in the stromal fraction correspond to osteo-chondrogenic progenitors that also express CD73 (Figure S2B). During the onset of endochondral ossification (E14.5–15.5), the four PαS subpopulations detected in adult long bones become apparent (Figures 5C and 5F, compare...
with Figure 4A). Already at this early stage, PαS cells are detected in the developing perichondrium (Figure S2C). Around birth (E18.5 and P2), the CD90posCD73hi PαS subpopulation is most abundant (Figures 5C and 5F). From W4 onward, the two CD90pos PαS subpopulations become most prominent as they increase to ~75% of all PαS (Figures 5C and 5F). The maximum numbers of PαS cells can be isolated from mouse long bones at early postnatal stages (P1–4: ~12–20 × 10^3 cells), while their numbers decrease progressively thereafter (~6.5 × 10^3 cells in adult mice, Figure 5G). Furthermore, the fraction of Sox9-GFP-positive cells remains rather constant in all four PαS subpopulations during fetal long bone development, but increases to ~70% by puberty around W4 (Figure 5D).

**In Vitro Tri-lineage Differentiation Potential of the Four PαS Subpopulations**

As PαS cells are most prominent at P2 (Figure 5G) and the chondrogenic and osteogenic programs are both active, the four subpopulations were isolated from limb long bones to assess their *in vitro* clonogenic and tri-lineage differentiation potential (Figures S3 and 6). Clonal analysis establishes that the clonogenic potential of the two CD90pos subpopulations is ~2-fold higher than one of the CD90neg subpopulations during fetal long bone development, but increases to ~70% by puberty around W4 (Figure 5D).
Figure 6. *In Vitro* Tri-lineage Differentiation Potential of the PaS Subpopulations

The four PaS subpopulations were sorted from hindlimb long bones of newborn mice (P2). Cells were briefly expanded and chondrogenic, osteogenic, and adipogenic differentiation using equal numbers of cells from all PaS subpopulations were induced in the appropriate differentiation media. Controls were maintained in expansion medium (see Supplemental Experimental Procedures).

(A) Chondrogenic differentiation was assayed after 5 days: (i) Sox9 and Col2a1 transcript levels and (ii) the distribution of SOX9 and collagen type 2 (COL2) proteins are shown.

(B) Osteogenic differentiation was assayed after 21 days. (i) Osterix (Osx) and Osteocalcin (Oc) transcript levels. (ii) The extent of mineralization was detected by Alizarin red staining and the OSX protein distribution analyzed by immunofluorescence.

(C) Adipogenic differentiation was analyzed molecularly after 5 days. (i) Pparγ2 and Fabp4 transcript levels (marking pre-adipocytes and adipocytes). (ii) The peroxisome proliferator-activated receptor γ (PPARγ) protein distribution was analyzed by immunofluorescence. Lipid droplets were revealed by Nile red staining after 10 days of differentiation.

Transcript levels were normalized to Rpl-19 transcripts. Nuclei were counterstained with DAPI (blue). n ≥ 2 independent experiments were analyzed per data point and yielded identical results. Results in (i) are presented as averages ± SD. Scale bars, 100 μm.
Figure 7. In Vitro Chondrogenic and In Vivo Bone-Forming Potential of CD90^{pos} and CD90^{neg} PαS Subpopulations

(A) Scheme showing the experimental setup for engraftment of cartilage constructs into nude mice. FACS, fluorescence-activated cell sorting; s.c., subcutaneous.

(B) Total PαS and CD90^{pos} and CD90^{neg} PαS subpopulations were isolated from long bones of newborn mice. Following 1 week of expansion, the different cells were seeded into collagen type 1 (COL1) matrices and cultured for 1 week in chondrogenic differentiation medium (experimental samples) or expansion medium (controls). 3D cartilage constructs were analyzed for morphology (blue) and chondrogenesis by Safranin O (red staining), and the SOX9 (red) and COL2 (green) distribution determined by immunofluorescence. Scale bars, 100 μm.

(C) 3D cartilage constructs were implanted subcutaneously into adult nude mice and retrieved 8 weeks later to assess morphology (blue) and cartilage by Safranin O red staining. Note the absence of cartilage in all samples. No ossicles developed in control implants (see B and data not shown). Frequencies of ossicles with bone structures: PαS constructs n = 5/5; CD90^{pos} PαS constructs n = 5/5; CD90^{neg} PαS constructs n = 2/5. Scale bars, 200 μm (see also Figure S4).

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PzS subpopulations (Figure S3A). However, cell clones derived from all four PzS subpopulations undergo proliferation arrest during in vitro expansion (Figure S3B and data not shown), which precludes generation of sufficient cells for tri-lineage differentiation analysis. Therefore, equal numbers of freshly isolated cells from all four PzS subpopulations were only briefly expanded prior to inducing differentiation into chondrogenic, osteogenic, and adipogenic cells (tri-lineage differentiation, Figure 6; for details see Supplemental Experimental Procedures).

Culturing cells in chondrogenic differentiation medium resulted in upregulation of Sox9 and Col2a expression in all four PzS subpopulations (Figure 6A). However, the Sox9 and Col2a expression levels are much higher in the two CD90neg PzS subpopulations (CD90negCD73int and CD90negCD73hi, Figure 6A). In agreement, the two CD90neg cell populations display a much higher osteogenic and adipogenic differentiation potential than CD90pos cells (Figures 6B and 6C). The in vitro tri-lineage differentiation potential of the two CD90neg subpopulations (CD90negCD73int and CD90negCD73hi) is lower, but it is important to note that these cells are able to initiate both chondrogenic and adipogenic differentiation programs as revealed by transcriptional upregulation of specific markers for these lineages (Figures 6A and 6C). In osteogenic culture conditions, the Osr and Osteocalcin (Oc) expression is upregulated in both CD90pos subpopulations (Figure 6Bi), but no OSX protein and mineralization (visualized by Alizarin red staining) are detected (Figure 6Bi). These results indicate that while the CD90neg PzS cells possess robust tri-lineage differentiation potential upon induction in culture, the CD90pos PzS cells initiate differentiation along all three lineages but fail to undergo complete adipogenic and osteogenic differentiation in 2D cultures (Figure 6).

**Cartilage Derived from CD90pos PzS Cells Is Remodeled In Vivo into Bone Organoids Attracting Host-Derived Angiogenesis and Hematopoiesis**

The in vivo differentiation and endochondral-bone-forming potential of CD90neg and CD90pos PzS cells isolated from newborn mice was assayed by subcutaneous engraftment of engineered 3D cartilage tissue implanted into nude mice (Figure 7). These two rather than all four PzS subpopulations were assayed, as the respective in vitro differentiation of the two CD90neg and two CD90pos subpopulations is very similar (Figure 6). Following flow-cytometric isolation of parental PzS cells and its CD90neg and CD90pos subpopulations, cells were briefly expanded, seeded into collagen type 1 (COL1) matrices, and cultured in chondrogenic medium (Figure 6A, see Experimental Procedures). After 1 week, the cartilage scaffolds generated by the three different cell populations were either analyzed (Figure 7B) or implanted subcutaneously into athymic nude mice (Figure 7C).

Safranin O staining of matrices and detection of the SOX9 and COL2 proteins after 2 weeks showed that all three cell populations are able to produce cartilage in COL1 matrices (Figure 7B). In particular, it appears that the chondrogenic differentiation potential of CD90pos PzS cells is significantly enhanced by culturing them in 3D COL1 matrices (Figure 7B, compare with Figure 6Aii).

Eight weeks after subcutaneous implantation, the three types of scaffolds were explanted and histological analysis established that all cartilage tissues engineered from parental PzS cells and the CD90pos PzS subpopulation had formed ossicles with bone marrow cavities (n = 5/5, Figure 7C). In contrast, bone formation by CD90neg PzS-based constructs is less efficient, as small ossicles with a poorly developed bone marrow compartment formed in only two of the five implants (Figure 7C). The absence of glycosaminoglycans (normally stained in red by Safranin O) establishes that the cartilage extracellular matrix was not maintained in vivo. Indeed, neither COL2 nor Aggrecan were detected in the remodeled explants (data not shown). As a significant fraction of PzS cells comprises SOX9-positive cells (Figure 5D), we analyzed the SOX9 distribution in the explants by immunofluorescence (Figure S4). Few scattered SOX9-positive cells are present in explants derived from PzS cells and

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**D** Immunofluorescence analysis of the different constructs after 8 weeks in vivo differentiation to detect OSX-positive cells (red) and CD31-positive endothelial cells (green, indicative of angiogenesis). Sections were counterstained with DAPI (blue) to reveal nuclei. The enlarged regions 1 and 2 are shown in the right panels. Scale bars, 500 μm (low magnification) and 100 μm (high magnification).

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**E** Immunofluorescence analysis of ossicles retrieved after implanting DsRed-positive CD90pos PzS cartilage constructs (8 weeks). Left panels show that the CD45-positive hematopoietic cells are host derived, as they are not expressing DsRed. Right panels show that the CD31-positive endothelial cells are also host derived. Scale bars, 100 μm (upper panels) and 10 μm (middle and lower panels).

**F** Sections through the bone marrow of CD90pos PzS ossicles. Immunofluorescence analysis reveals Gr1-positive granulocytes and B220-positive cells (immature and mature B lymphocytes). Nuclei were counterstained with DAPI (blue). Scale bars, 100 μm (low magnification) and 10 μm (high magnification).

**G** Co-localization of CD45 and SCA-1 (yellow) labels HSCs and multipotent progenitors. Co-expressing cells were observed near the endosteeum (arrowhead in the left panel). Nuclei were stained with DAPI. Scale bars, 100 μm (low magnification) and 10 μm (high magnification).

All results are representative of n ≥ 3 independent samples.
CD90<sup>pos</sup>P<sub>S</sub> chondrogenic scaffolds, while abundant SOX9-positive cells remain in CD90<sup>neg</sup>P<sub>S</sub> explants (Figure S4). In fact, this analysis showed that cartilage was not maintained in any of the constructs generated using the three different cell populations. This is likely due to the fact that the milieu of the host does not support cartilage maintenance, which depends on inhibition of VEGF signaling (Chan et al., 2015).

The distribution of CD31-positive endothelial and OSX-positive cells (Maes et al., 2010; Ono et al., 2014a) was analyzed on sections to gain insight into the extent to which the endochondral ossification had processed (Figure 7D). Cells expressing these markers are abundant in both P<sub>S</sub> cells and CD90<sup>pos</sup>P<sub>S</sub> ossicles, but the distribution of CD31-positive and OSX-positive cells is much more organized in ossicles derived from CD90<sup>pos</sup>P<sub>S</sub> than in the ones using unfractionated P<sub>S</sub> (Figure 7D). This correlates well with the larger and well-structured bone marrow compartment observed in CD90<sup>pos</sup>P<sub>S</sub> ossicles, which are reminiscent of bone organoids (Figure 7C). In contrast, fewer OSX-positive and no CD31-positive cells are detected in CD90<sup>neg</sup>P<sub>S</sub> explants (Figure 7D). These observations, together with the presence of a large number of SOX9-positive cells (Figure S4), suggests that endochondral ossification was arrested at an early step during remodeling of CD90<sup>pos</sup>P<sub>S</sub> implants.

To determine whether the endothelial and OSX-positive cells were derived from implant or host, we isolated CD90<sup>pos</sup>P<sub>S</sub> cells from newborn mice expressing DsRed ubiquitously (Vintersten et al., 2004) and used them to generate cartilage constructs for implantation (Figure 7A). After 8 weeks, explants were analyzed by immunofluorescence. Donor-derived DsRed-positive cells are located in the compact bone and in the bone marrow in close contact with host-derived endothelial and hematopoietic cells (Figure 7E). In contrast, all hematopoietic (CD45) and endothelial (CD31) cells lack DsRed, which establishes that they are recruited from the host (Figure 7E). Further analysis of the hematopoietic compartment showed that CD45<sup>pos</sup> cells encompass descendants of the common myeloid (granulocytes Gr1<sup>pos</sup>) and common lymphoid progenitors (immature and mature B cells detected by B220, Figure 7F). Rare CD45 SCA-1 double-positive cells are detected near the endosteme, which is indicative of multipotent hematopoietic progenitors (Seita and Weissman, 2010). Our results establish that expanding P<sub>S</sub> CD90<sup>pos</sup> shortly in culture and differentiating them in 3D COL1 matrices results in cartilage constructs that are very efficiently remodeled into bone organoids in vivo (Figure 7C). In particular, the engrafted constructs attract host-derived endothelial cells to establish angiogenesis and generate a niche to recruit and maintain host-derived multipotent HSCs for hematopoiesis (Figures 7D–7G).

**DISCUSSION**

Flow-cytometric analysis was used to study the ontogeny of different mesenchymal progenitor populations during mouse limb and long bone development and homeostasis. We show that the majority of Lin<sup>neg</sup> mesenchymal cells during embryonic limb and fetal long bone are P<sub>S</sub>CD51 cells (Pinho et al., 2013), while their numbers drop drastically after birth. A small fraction of Prx1-expressing P<sub>S</sub>CD51 cells persists into adulthood. This is in support of P<sub>S</sub>CD51 cells retaining progenitor characteristics. Most importantly, P<sub>S</sub>CD51 cells encompass at least three distinct cell populations: P<sub>S</sub> cells (Morikawa et al., 2009), CD200<sup>pos</sup> cells that contain all mSSCs (Chan et al., 2015), and at least one additional population, SCA-1<sup>neg</sup>CD200<sup>neg</sup>P<sub>S</sub>CD51 cells. These P<sub>S</sub>CD51DN cells express the highest levels of Cxcl12, which indicates that they might encompass/correspond to the adipo-osteogenic CAR progenitors needed for maturation of B lymphocytes (Greenbaum et al., 2013; Omatsu et al., 2010; Sugiyama et al., 2006). This is in line with the recent observation that CAR cells are derived from the P<sub>S</sub> population (Hu et al., 2016) and the fact that human P<sub>S</sub>CD51 cells isolated from fetal bone marrow are self-renewing, possess multilineage potential, and provide HSC niche functions (Pinho et al., 2013). In addition, the different mesenchymal cell populations express Lepr, which is interesting as Lepr-positive mesenchymal progenitors are a main source of bone formed by adult bone marrow (Zhou et al., 2014a). Finally, we show that the most abundant stromal cells from adult bones are P<sub>z</sub> cells. However, as these P<sub>z</sub>-positive cells neither express Prx1 nor any of the other osteo-chondrogenic markers analyzed in a robust manner, they likely correspond to fibroblasts given their poor survival in culture (G.N. and R.Z., unpublished data).

Our results establish the SCA-1<sup>pos</sup>P<sub>S</sub> and the CD200<sup>pos</sup>mSSCs are mutually exclusive mesenchymal progenitors with distinct developmental origins. Within the P<sub>S</sub>CD51 mesenchymal progenitors, SCA-1<sup>pos</sup> cells are detected much earlier than CD200<sup>pos</sup> cells in mouse limb buds. Others have proposed that the CD200<sup>pos</sup>mSSCs are related to the progenitors that participate in endochondral bone formation during limb bud development, postnatal bone growth, and fracture healing (Cervantes-Diaz et al., 2016; Chan et al., 2015; Serafini et al., 2014; Yang et al., 2014; Zhou et al., 2014b). In contrast, the Sca-1-expressing mesenchymal progenitors are detected in early limb buds surrounding the chondrogenic anlagen. Contrary to bulk of limb bud mesenchymal progenitors, SCA-1<sup>pos</sup> cells are
not significantly expanded prior to the onset of endochondral ossification (G.N. and R.Z., unpublished data). After birth, PzS cells continue to express Prx1 and low levels of Cxcl12 and Lepr, but not Col2a1, Mmp13, and Osx (Greenbaum et al., 2013; Morikawa et al., 2009; Ono et al., 2014b). At this stage it is unclear whether the SCA-1pos progenitors present in early mouse limb buds give rise to definitive PzS cells.

We also show that PzS cells are not homogeneous, but consist of four subpopulations that become apparent during the onset of endochondral ossification. The highest numbers of PzS cells are detected perinatally and in newborn mice; coinciding with abundant chondrogenic and osteogenic activity, peak of endothelial cell numbers, and migration of fetal HSCs from liver to bone marrow (Ono et al., 2014a; Trumpp et al., 2010). While all four subpopulations display CFU-F frequencies similar to that of the parental PzS population (Morikawa et al., 2009), they cannot be extensively expanded in culture (this study). In agreement with the major chondrogenic activity during embryonic and early postnatal long bone development, the two CD90pos subpopulations represent the bulk of PzS cells during this period and possess the best tri-lineage differentiation potential in vitro. Therefore, it was unexpected that CD90neg PzS cartilage constructs are not remodeled into bone organoids, but appear arrested at an early stage. This suggests that 3D cartilage constructs generated from CD90neg PzS cells either lack osteogenic progenitors and/or that chondrogenesis did not progress to hypertrophy, which is necessary to trigger endochondral ossification (Long and Ornitz, 2013).

The ratio between CD90neg and CD90pos cells reverses in long bones around 2 weeks after birth as the CD90pos PzS subpopulation becomes predominant. This switch occurs as the migration of HSCs is complete and bone marrow homeostasis is achieved (Trumpp et al., 2010). CD90pos PzS cells initiate, but do not complete tri-lineage differentiation in 2D culture, while seeding into 3D COL1 matrices results in efficient cartilage production. Most strikingly, CD90pos PzS cells have the highest CFU-F potential in culture, and cartilage constructs derived from these cells are efficiently remodeled into bone organoids in vivo. These bone organoids contain a well-structured marrow with a host-derived hematopoietic and vascular system. In particular, donor-derived Osxpos cells resembling perinatal mesenchymal stromal progenitors (Liu et al., 2013; Maes et al., 2010; Mizoguchi et al., 2014; Ono et al., 2014b) are present in proximity to host-derived endothelial and hematopoietic cells. Most importantly, rare CD45posSCA-1pos cells are detected close to the endostium, indicating that they correspond to short-term self-renewing HSCs. These findings agree with previous studies showing that PzS cells are required to maintain long-term self-renewing HSCs (Greenbaum et al., 2013; Hu et al., 2016; Morikawa et al., 2009).

Our study defines the emergence and relationships among the most relevant MSC-like populations in mice. In addition, we identify distinct PzS subpopulations and show that one of them, the CD90pos PzS subpopulation, has the potential to differentiate into cartilage that is remodeled into bone organoids with a functional marrow in mice. As mouse PzS cells are contained within the PzCD51 population, our study could pave the way to identify the orthologous cells in humans, which may have important therapeutic implications for cartilage and bone tissue engineering and their co-transplantation with HSCs in human patients.

**EXPERIMENTAL PROCEDURES**

A detailed description of all procedures is included in Supplemental Experimental Procedures.

**Mouse Strains**

All studies using mice were performed strictly in adherence with Swiss law, the 3R principles, and the Basel Declaration. All animal studies were approved by the cantonal animal welfare and ethics committee (licenses no. 1951 to R.Z. and no. 1797 to I.M.). Personnel performing animal studies are trained and licensed according to FELASA standards. The persons performing surgery (subcutaneous implantation) have been specially trained. Unless indicated otherwise, mice of both sexes were used for analysis. The Prx1-Cre (Logan et al., 2002), DsRed (mouse strain generated using DsRed.T3-expressing embryonic stem cells; Vintersten et al., 2004), inducible β-Act-GFP (Jagle et al., 2007), and Sox9IRES-EGFP (Sox9-GFP, Chan et al., 2011) mouse strains were kept in a C57BL/6 genetic background. C57BL/6 and CD1 nude mice were purchased from Janvier and Charles River Laboratories, respectively.

**Isolation of Mesenchymal Cells from Limb Buds and Long Bones for Flow-Cytometric Analysis**

Cell suspensions were prepared from mouse embryonic limb buds and long bones for flow-cytometry analysis and sorting as described by Houlihan et al. (2012). The modifications necessary to adapt the protocol to the different developmental stages analyzed are described in Supplemental Experimental Procedures.

**Subcutaneous Engraftment of 3D Cartilage Scaffolds**

PzS and CD90pos and CD90posPzS subpopulations were sorted by flow cytometry from mouse hindlimb long bones at postnatal day 2 (P2) and expanded in vitro for 5–7 days under normoxic conditions (21% O2, 5% CO2; Supplemental Experimental Procedures). Then 100,000 cells (20,000 cells/μL) were seeded per COL1-matrix (Avitene Ultrafoam Collagen Sponge; C.R. Bard, USA) and cultured in expansion medium overnight. Half of the matrices were continuously cultured in expansion medium to serve as controls, while the others were cultured in chondrogenic

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differentiation medium for 7 days (Supplemental Experimental Procedures). After this, one set of COL1-constructs per cell type was used for histological analysis by Von Kossa staining, Safranin O staining, and immunofluorescence. The others were subcutaneously implanted into adult nude CD1 mice (weeks 12–15) as previously described (Scotti et al., 2010). Constructs were retrieved 8 weeks later and analyzed by histology and immunofluorescence (Supplemental Experimental Procedures and Scotti et al., 2013).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.08.007.

AUTHOR CONTRIBUTIONS

G.N and S.J. performed all the flow-cytometric studies and most of the other analysis. R.R. performed the in situ hybridization. A.B. and A.H. performed the implantation studies in nude mice, and the analysis was done together with S.J. and G.N. Adipogenic differentiation assays were performed by D.I.R. and S.J., and T.L. provided the Sox9<sup>IRESGFP</sup> (Sox9-GFP) mice generated in his group prior to publication. R.Z., I.M., and G.N conceived and supervised the studies. I.M and R.Z. acquired the necessary funding. R.Z and G.N. wrote the manuscript with input from all authors.

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