SUMMARY

Mesenchymal stem and progenitor cells (MSPCs) contribute to bone marrow (BM) homeostasis by generating multiple types of stromal cells. MSPCs can be labeled in the adult BM by Nestin-GFP, whereas committed osteoblast progenitors are marked by Osterix expression. However, the developmental origin and hierarchical relationship of stromal cells remain largely unknown. Here, by using a lineage-tracing system, we describe three distinct waves of contributions of Osterix+ cells in the BM. First, Osterix+ progenitors in the fetal BM contribute to nascent bone tissues and transient stromal cells that are replaced in the adult marrow. Second, Osterix-expressing cells perinatally contribute to osteolineages and long-lived BM stroma, which have characteristics of Nestin-GFP+ MSPCs. Third, Osterix labeling in the adult marrow is osteolineage-restricted, devoid of stromal contribution. These results uncover a broad expression profile of Osterix and raise the intriguing possibility that distinct waves of stromal cells, primitive and definitive, may organize the developing BM.

INTRODUCTION

The bone marrow (BM) environment is composed of multiple cell types, most of which are thought to be derived from mesenchymal stem and progenitor cells (MSPCs) (Bianco et al., 2013; Caplan, 1991; Frenette et al., 2013). Stromal progenitor activity in the BM was initially isolated from clonal populations of fibroblastic colony-forming units (CFU-F) that exhibit self-renewal and the capacity to differentiate into the major mesenchymal lineages (Friedenstein et al., 1968; Mendez-Ferrer et al., 2010; Sacchetti et al., 2007). Although surface markers have been suggested to mark MSPCs (Dominici et al., 2006), these were based on cultured stromal cells, but not on prospectively isolated native stroma and lack specificity to identify native bone marrow MSPCs (Bianco et al., 2013). In the mouse BM, transgenic mice expressing GFP under the Nestin promoter (Nes-GFP) select for MSPC activity and so do stromal cells with CD45−/Tie2−/CD90+/CD105+ phenotype (Chan et al., 2009), CXCL12 abundant reticular (CAR) cells (Omatsu et al., 2010), PDGFRα+Sca-1+ (Morikawa et al., 2009), CD51+PDGFRα+ (Pinho et al., 2013), and Prx-1-derived CD45−Ter119−PDGFRα+Sca-1+ populations (Greenbaum et al., 2013). There is evidence that these stromal cell populations display some significant overlap with each other and comprise important cellular constituents of the hematopoietic stem cell (HSC) niche. For example, Nes-GFP+ cells highly overlap with leptin receptor (Lepr)-expressing perivascular cells (Pinho et al., 2013), which were shown to be a major source of CXCL12 and stem cell factor (SCF) in the BM (Ding and Morrison, 2013; Ding et al., 2012). These reports thus suggest that MSPCs organize the BM environment by contributing to osteolineage cells and regulating HSC self-renewal and differentiation.

Additionally, other studies have suggested a role for osteoblasts as a constituent of the HSC niche. Gain- and loss-of-function approaches have shown that alterations in osteoblast numbers correlate with the number of HSCs (Cali et al., 2003; Visnjic et al., 2004; Zhang et al., 2003), although the correlation was not observed in other models (Kiel et al., 2007; Lymperi et al., 2008). Osteoblasts have been suggested to regulate the HSCs via secretion of angiopoietin-1 (Arai et al., 2004), osteopontin (Nilsson et al., 2005; Stier et al., 2005), and noncanonical Wnt signaling (Sugimura et al., 2012). However, the expression of these factors is not specific to osteoblasts and there is no evidence thus far that specific deletion of these factors in committed osteoblasts affects HSC maintenance.

One of the promoters expressed in the bone marrow thought to be specific to the osteolineage is Osterix (Osx), a transcription factor shown to be required for bone formation (Nakashima et al., 2002). During bone development, Osx+ osteoblast precursors appear around the perichondrium and subsequently migrate...
into the developing primary ossification center along with blood vessels, giving rise to mature osteoblast cells (Karsenty and Wagner, 2002; Maes et al., 2010). In the adult, Osx+ cells provide a transient source of osteoblasts (Park et al., 2012), implying the presence of a more primitive source sustaining osteoblast cells throughout the lifetime. Here, we show unexpectedly that Osx marks successive waves of progenitors during ontogeny, including bona fide MSPCs at the perinatal stage. In addition, our studies have uncovered temporally distinct stromal precursors, termed primitive and definitive stroma, that differentially contribute to skeletal development.

RESULTS AND DISCUSSION

Neonatal Osx+ Cells Give Rise to Long-Lived BM Stromal Cells

To trace lineages of Osx-expressing cells in the developing bone and BM, we generated double-transgenic Osx-CreERT2/ROSA26-loxP-stop-loxP-taTomato (iOsx/Tomato) reporter mice where cre expression in Osx+ cells can be induced at different developmental stages by administration of tamoxifen (Tam). Consistent with previous observations (Maes et al., 2010), Osx+ cells were labeled in the perichondrium 1 day after Tam injection in embryonic day 13.5 (E13.5) mice, before the formation of a BM cavity (Figure 1A). The iOsx expression was consistent with staining of the endogenous protein using an anti-Osx antibody confirming the specificity of transgenic expression (Figure S1A available online). After a chase of 2 weeks, Osx+ cell progeny (designated E13.5-iOsx/Tomato+ cells) were detected in the primary spongiosa adjacent to blood vessels (Figure 1B1) labeled by VE-cadherin and PECAM-1 staining and around the cortical bone (Figure 1B2). Osx+ cells labeled during fetal BM development gave rise to the full spectrum of osteoblast lineage cells in the growing bone (Maes et al., 2010). Interestingly, our results revealed that 2 weeks after labeling, E13.5-iOsx/Tomato+ cells were not only detected in bone tissues, but also in stromal cells that did not express osteocalcin, in contact with the vasculature (Figures 1B2 and S1C). However, following a chase of 13 weeks, the osteoblast lineage BM stromal cells were clearly decreased along with the longitudinal growth, suggesting that embryonic Osx+ progenitor cells transiently contribute to the developing bone and marrow stroma (Figures 1C and S1D).

We then marked Osx+ cells at postnatal day 5 (PND5; designated P5-iOsx/Tomato+ cells) and found that they included cells of the primary spongiosa and around the cortical bone, but not in the BM cavity 1 day after Tam injection (Figure 1D). The specificity of the iOsx-marked cells was also confirmed at this time point using anti-Osx antibody staining (Figure S1B). FACS analysis revealed that P5-iOsx/Tomato+ cells in the bone tissue were CD45+ Ter119− CD31− stromal cells (97.6% ± 0.4%) and that these cells were rarely detected in the BM 1 day after Tam injection (Figure S1E). However, after a chase of 3 weeks, P5-iOsx/Tomato+ osteoblasts and osteocytes were detected around the bone and, unexpectedly, P5-iOsx/Tomato+ stromal cells were also observed adjacent to blood vessels in the BM cavity (Figure 1E). In addition, the stromal cell labeling persisted for at least 32 weeks throughout the BM (Figure 1F: chase of 24 weeks; Figure S1F: chase of 32 weeks). FACS analysis of P5-iOsx/Tomato+ cells after a chase of 15 weeks indicated that they remained confined to the stromal CD45− Ter119− CD31− compartment (97.7% ± 0.4%; Figure S1G). Further analyses of the characteristics of the P5-iOsx/Tomato+ BM cells revealed that the cells became quiescent during bone growth (Figures S1H and S1I). The contribution of P5-iOsx/Tomato+ cells to the BM stroma increased in a time-dependent manner as seen by the progressive increased frequency within total stroma and their absolute number per femur (Figures S2A and S2B). By contrast, iOsx/Tomato mice pulsed in the adult stage (8 weeks of age) displayed restricted labeling to the bone tissues 1 day post-Tam injection (Figure 1G) and did not generate BM stromal cells after 2 or 7 weeks chase (Figures 1H and 1I). Consistent with recent studies (Park et al., 2012), iOsx labeling was reduced after 7 weeks (Figures 1G–1I), suggesting that iOsx marks nonself-renewing osteoprogenitors in the 8 weeks pulsed iOsx/Tomato mice. Indeed, after a chase of 12–15 weeks, a significantly higher number of iOsx/Tomato+ bone-lining cells was found in the P5 pulsed mouse bone tissue than in the 8-week-old pulsed mice (Figures S2C–S2E; 38.6% ± 6.5% versus 8.0% ± 1.4%, p < 0.05), and these cells were sustained for up to 32 weeks (Figure S1F), suggesting that the P5-iOsx/Tomato+ cells contain long-lived stromal cells that contribute to osteolineages. These stromal cells can self-renew in vitro because clonally expanded P5-iOsx/Tomato+ cells were able to form primary spheres in nonadherent culture conditions (Méndez-Ferrer et al., 2010) and when dissociated, could form secondary clonal spheres with a similar efficiency as the primary spheres (primary: 0.6% ± 0.1%; secondary: 0.5% ± 0.1%; Figures S2F and S2G). These results thus suggest that iOsx temporally marks at least three distinct mesenchymal precursor cells in the fetal, perinatal, and adult bone marrow and that the perinatal Osx-expressing cells, which become quiescent during bone growth, also exhibit self-renewal capacity.

P5-iOsx-Derived BM Stromal Cells Possess MSCP Characteristics

To test whether P5-iOsx/Tomato+ stromal cells contained MSCP activity, we generated Nes-Gfp/Osx/Tomato triple-transgenic mice. One day after Tam injection, a fraction of P5-iOsx/Tomato+ cells in the primary spongiosa and around the cortical bone area were Nes-GFP+ (Figure 2A), FACS analysis showed that 38.2% ± 2.2% of P5-iOsx/Tomato+ cells in the bone tissue expressed Nes-GFP (Figure 2C). However, most (84.9% ± 6.5%) of P5-iOsx/Tomato+ BM cells were Nes-GFP+ by histological and FACS analyses 15 weeks after Tam injection (Figures 2B and 2D). P5-iOsx/Tomato+ BM stromal cells were highly enriched in CFU-F activity (Figure 2E) whose colonies exhibited Tomato fluorescence (Figure 2F), thereby confirming that they were derived from Osx+ cells marked at P5. Furthermore, the clonally expanded P5-iOsx/Tomato+ BM cells exhibited trilineage differentiation potential (Figures 2G–2I). The persistence of perinatally marked iOsx stromal cells in the bone marrow is consistent with recently published data in which E14.5 Osx-expressing cells marked long-lived stroma (Liu et al., 2013). Thus, definitive MSPCs may be established late in gestation.

Recent studies reported that leptin receptor+ (Lepr)+ stromal cells contribute to HSC maintenance as a major source of SCF (Ding et al., 2012). Our recent analyses indicate that Lepr+ cells in the BM largely overlap with Nes-GFP+ cells (Pinho et al., 2013). We then marked Lepr+ cells at postnatal day 5 (PND5; designated P5-Lepr+ cells) and found that they included cells of the primary spongiosa and around the cortical bone, but not in the BM cavity 1 day after Tam injection (Figure 1D). The specificity of the cLepr+ cells was also confirmed at this time point using anti-Lepr antibody staining (Figure 3A). FACS analysis revealed that P5-cLepr+ cells in the bone tissue were CD45+ Ter119− CD31− stromal cells (97.6% ± 0.4%) and that these cells were rarely detected in the BM 1 day after Tam injection (Figure S3E). However, after a chase of 3 weeks, P5-cLepr+ osteoblasts and osteocytes were detected around the bone and, unexpectedly, P5-cLepr+ stromal cells were also observed adjacent to blood vessels in the BM cavity (Figure 3E). In addition, the stromal cell labeling persisted for at least 32 weeks throughout the BM (Figure 3F: chase of 24 weeks; Figure S3F: chase of 32 weeks). FACS analysis of P5-cLepr+ cells after a chase of 15 weeks indicated that they remained confined to the stromal CD45− Ter119− CD31− compartment (97.7% ± 0.4%; Figure S3G). Further analyses of the characteristics of the P5-cLepr+ BM cells revealed that the cells became quiescent during bone growth (Figures S3H and S3I). The contribution of P5-cLepr+ cells to the BM stroma increased in a time-dependent manner as seen by the progressive increased frequency within total stroma and their absolute number per femur (Figures S3A and S3B). By contrast, cLepr+/Tomato mice pulsed in the adult stage (8 weeks of age) displayed restricted labeling to the bone tissues 1 day post-Tam injection (Figure 3G) and did not generate BM stromal cells after 2 or 7 weeks chase (Figures 3H and 3I). Consistent with recent studies (Park et al., 2012), cLepr+ labeling was reduced after 7 weeks (Figures 3G–3I), suggesting that cLepr+ marks nonself-renewing osteoprogenitors in the 8 weeks pulsed cLepr+/Tomato mice. Indeed, after a chase of 12–15 weeks, a significantly higher number of cLepr+/Tomato+ bone-lining cells was found in the P5 pulsed mouse bone tissue than in the 8-week-old pulsed mice (Figures S3C–S3E; 38.6% ± 6.5% versus 8.0% ± 1.4%, p < 0.05), and these cells were sustained for up to 32 weeks (Figure 3F), suggesting that the P5-cLepr+/Tomato+ cells contain long-lived stromal cells that contribute to osteolineages. These stromal cells can self-renew in vitro because clonally expanded P5-cLepr+/Tomato+ cells were able to form primary spheres in nonadherent culture conditions (Méndez-Ferrer et al., 2010) and when dissociated, could form secondary clonal spheres with a similar efficiency as the primary spheres (primary: 0.6% ± 0.1%; secondary: 0.5% ± 0.1%; Figures 3F and S3G). These results thus suggest that cLepr temporally marks at least three distinct mesenchymal precursor cells in the fetal, perinatal, and adult bone marrow and that the perinatal Osx-expressing cells, which become quiescent during bone growth, also exhibit self-renewal capacity.
Figure 1. Lifelong Contribution of Osx+ Cells to the BM Cells in Developing Bones

Z stack confocal images of thick bone sections of Osx/Tomato mice administered with tamoxifen (Tam) at embryonic day 13.5 (E13.5) (A–C), postnatal day 5 (P5) (D–F), and 8-week-old (G–I) mice analyzed at the indicated periods. Bone sections were stained with VE-cadherin (VE-Cad), PECAM-1 antibodies (green), and Hoechst 33342 (blue). Right panels are magnified views of the boxed areas. Scale bars represent 100 μm. Arrows: Osx-derived Tomato+ (Osx/Tomato+) stromal cells. Arrowheads: Osx/Tomato+ osteolineage cells. See also Figures S1 and S2.
Figure 2. Osx+ Cells in the Neonatal Bone Give Rise to Nes-GFP+ MSPCs
Analysis of Nes-Gfp/iOsx/Tomato mice administered with tamoxifen (Tam) at P5.
(A and B) Z stack confocal images of thick bone sections at 1 day (A) and 15 weeks (B) after Tam injection. Bone sections were stained with VE-cadherin (VE-Cad) and PECAM-1 antibodies (white). Right panels are magnified confocal images within the area defined by the rectangle. Arrows: Nes-GFP and iOsx-derived Tomato (iOsx/Tomato) double-positive cells.

Developmental Origin of Bone Marrow MSPCs
Developmental Cell

Developmental Origin of Bone Marrow MSPCs

Based on these results, we analyzed the expression of Lepr in P5-iOsx/Tomato+ BM cells with an anti-Lepr antibody. We found that most P5-iOsx/Tomato and Nes-GFP double-positive cells at 15 weeks after Tam injection expressed Lepr by immunostaining (Figure 2J) and flow cytometry (Figure 2K; 78% ± 4.5%), and this population also expressed the MSPC markers PDGFRα (~89%) and PDGFRβ (~83%) (Figure 2K) (Crisan et al., 2008; Morikawa et al., 2009; Pinho et al., 2013). On the other hand, the expression of Lepr was very low in P5-iOsx/Tomato+ cells at 1 day after Tam injection (Figure S2H), suggesting that the Lepr expression increases in the P5-iOsx/Tomato+ BM cells during bone maturation. Interestingly, E13.5-iOsx/Tomato+ BM cells also express Nes-GFP, Lepr, PDGFRα, and PDGFRβ after a chase of 2 weeks (Figures S2I and S2J). Ono et al. (2014) suggest that some of the Osx+ cells labeled at E12.5 overlap with Nes-GFP+ cells. Collectively, these results thus support the idea that Osx+ cells in the neonatal bone marrow are the precursors of Nes-GFP+ Lepr+ MSPCs in the adult BM, although whether Osx+ cells were initially Nes-GFP+ cannot be excluded.

Lepr-cre-Derived BM Stromal Cells Contribute to the Osteolineage in Adulthood

Lepr-marked stromal cells were suggested to be restricted to perivascular areas and do not contribute to osteolineage cells (Ding et al., 2012). Because we found that Lepr was expressed in Nes-GFP+ MSPCs, we analyzed the contribution of Lepr+ cells to bone tissues in Nes-GFP/Lepr-cre/Tomato triple transgenic mice. Consistent with the above antibody staining experiments (Figures 2J and 2K), Lepr-cre-derived Tomato+ cells (hereafter Lepr/Tomato+) expressed Nes-GFP, PDGFRα, and PDGFRβ (Figure S3A). Furthermore, clonally expanded Lepr/Tomato+ cells exhibited trilineage differentiation capacity (Figures S3B–S3D), indicating that these populations highly overlap with the P5-iOsx/Tomato+ cells in the BM. During early limb development, Lepr/Tomato+ cells were not present in the primary ossification center at E15.5, whereas Nes-GFP+ and Osx+ cells were expressed at this time in this location (Figure 3A). Lepr/Tomato+ cells were first detected in the primary spongioza and in the periosteous of the bone tissue at E17.5 and subsequently were present throughout the BM cavity in 1-week-old mice (Figure 3A). Immunofluorescence analyses revealed that Lepr/Tomato+ cells were positive for Nes-GFP and Osx in the primary spongioza in 1-week-old mice (Figure S3E). By FACS analysis, most (~92%) of Lepr/Tomato+ cells in the BM were also positive for Nes-GFP at 1 week of age (Figure S3F).

Next, we analyzed bone tissues of 3- and 15-week-old Lepr-cre/Tomato mice to investigate the osteolineage contribution of Lepr/Tomato+ BM stromal cells. In 3-week-old animals, Lepr/Tomato+ cells were distributed throughout the BM cavity, but were not present along the endosteum (Figure 3B, left). By contrast, in the 15-week-old bone tissues, Lepr/Tomato+ cells were observed not only in the BM cavity, but also along the cortical bone (Figure 3B, right). Immunofluorescence staining showed that Lepr/Tomato+ cells in the bone tissue were osteocalcin- and dentin matrix protein 1 (DMP1)-expressing mature osteoblasts and osteocytes, respectively (Figures 3C and 3D). Lepr/Tomato+ osteolineage cells were also observed in the trabecular bone of 15-week-old mice (Figure S3G). To examine whether Lepr/Tomato+ mature osteolineage cells express Lepr, we performed immunofluorescence staining with anti-Lepr antibody in Col1(2.3)-GFP and Lepr-cre/Tomato mice. We found that Col1(2.3)-GFP+ mature osteoblasts and Lepr/Tomato+ osteolineage cells were negative for Lepr (Figures 3E and 3F). In addition, Lepr mRNA was not detectable by quantitative real-time PCR in the osteoblasts (Figures S3H–S3J). These results suggest that Lepr/Tomato+ mature osteolineage cells do not autonomously express Lepr, but are descendant of Lepr+ precursors. Further, these data indicate that BM cells marked by Lepr-cre highly overlap with P5-iOsx/Tomato+ BM stromal cells that are a permanent source of osteoprogenitors contributing to adult bone homeostasis.

P5-iOsx-Derived BM Stromal Cells Contribute to Tissue Regeneration after Injury

Although MSPCs are thought to have the potential to differentiate into osteoblasts and adipocytes (Takada et al., 2009), their contribution to these lineages in vivo remains unclear. To test whether Lepr/Tomato+ and P5-iOsx/Tomato BM stromal cells had the potential to generate adipocytes in vivo, we challenged 8-week-old Lepr-cre/Tomato and 15-week-old iOsx/Tomato mice pulsed at P5 with 6 Gy irradiation, an injury known to induce fatty infiltration in the BM (Bryan et al., 1979). Adipocytes, identified by staining with BODIPY (493/503), a dye that stains lipid droplets (Spangenberg et al., 2011) and perilipin, an essential protein for adipogenesis (Martinez-Botas et al., 2000) were dramatically increased in the BM 6 days after irradiation (Figures 4A–4F and S4A). Importantly, these adipocytes were
Figure 3. Lepr-cre-Derived BM Stromal Cells Give Rise to Bone-Lineages in the Adult Stage

(A) Z stack confocal images of thick bone sections of Nes-Gfp/Lepr-cre/Tomato mice in the indicated stages, stained with Osx antibody (white). *Primary ossification center. Arrows: Lepr-cre-derived Tomato+ (Lepr/Tomato+) cells in the epiphysis. Arrowheads: Lepr/Tomato+ cells in the periosteum.

(B) Z stack confocal images of thick bone sections of Lepr-cre/Tomato mice in the indicated stages. Arrows: Lepr/Tomato+ osteoblasts. Arrowheads: Lepr/Tomato+ osteocytes.

(C and D) Confocal images of bone tissues from 15-week-old Lepr-cre/Tomato mice stained with osteocalcin (green) (C) and DMP1 antibodies (green) (D). (E) Z stack confocal images of thick bone sections in 6-week-old Col1(2.3)-Gfp mice stained with Lepr antibody (red). Right: magnified view the area of around the cortical bone. Arrows: Col1(2.3)-GFP-positive mature osteoblasts.

(F) Z stack confocal images of thick bone sections in 15-week-old Lepr-cre/Tomato mice stained with Lepr antibody (white). Arrows: Lepr/Tomato+ osteocytes. Arrowheads: Lepr/Tomato+ osteoblasts. Nuclei were detected by Hoechst 33342 (blue).

Scale bars represent 500 μm (E, left panel), 200 μm (B), 100 μm (A), 30 μm (E, right panels) and (F), and 10 μm (C) and (D). See also Figure S3.
Figure 4. P5-iOsx-Derived BM Stromal Cells Contribute to Tissue Remodeling after Tissue Injury

(A–F) Z stack confocal (A and D) and confocal (B, C, E, and F) images of thick bone sections at day 6 postirradiation stained with BODIPY (493/503) (green) (A, B, D, and E) or Perilipin antibody (green) (C and F) from 8-week-old Lepr-cre/Tomato mice (A–C) or P5-labeled iOsx/Tomato mice after 15 weeks chase (D–F). Arrows: Tomato+ adipocytes.

(G–L) Images of bone sections at day 8 post-bone fracture from P5-labeled iOsx/Tomato mice after 32 weeks chase (G–I) or 15-week-old Lepr-cre/Tomato mice (J–L). Serial sections stained with Toluidine blue (G and J). Z stack confocal (H and K) and confocal (I and L) images of thick bone sections stained with

(legend continued on next page)
also Tomato+ (Figures 4B, 4C, 4E, and 4F). We next intercrossed Lepr-cre with an inducible diphtheria toxin (DT) receptor (iDTR) line and examined the effect of the depletion of Lepr+ cells on the adipogenesis. The number of irradiation-induced adipocytes was significantly decreased by the deletion of Lepr+ cells (Figures S4B–S4E). However, no effect was observed in the number of adipocytes after DT injection in the control groups (Figures S4D and S4E). These results thus suggest that irradiation-induced adipocytes are derived from Lepr/Tomato+ BM cells as well as P5-iOsx/Tomato+ BM stromal cells. Interestingly, consistent with our results, a recent report has shown that the deletion of Wnt/β-catenin-signaling (an inhibitory signal of adipogenesis) in Osx-expressing cells leads to increased numbers of BM adipocytes (Song et al., 2012).

To determine whether P5-iOsx/Tomato+ and Lepr/Tomato+ BM stromal cells participate in the regenerative healing process after bone fracture, we used the semistabilized tibia fracture model (Maes et al., 2006) and subjected 32-week-old P5 pulsed iOsx/Tomato and 15-week-old Lepr-cre/Tomato mice to bone fracture. Tomato label-retaining chondrocytes were not observed in P5 pulsed iOsx/Tomato mice after a chase for 3 weeks (data not shown). Eight days after bone fracture, P5-iOsx/Tomato+ cells were observed in the newly formed chondrogenic area of the fracture callus (Figures 4G and 4H). Some of the P5-iOsx/Tomato+ cells were also positive for the chondrocyte marker Sox9 indicating that P5-iOsx/Tomato+ cells differentiated into chondrocytes and contributed to the bone fracture healing process (Figures 4G–4I). Lepr/Tomato+ cells were also detected as Sox9-positive cells in the fracture callus in Lepr-cre/Tomato mice (Figures 4J–4L). We confirmed that Lepr/Tomato+ cells in the fracture callus did not express Lepr with anti-Lepr antibody (Figure S4F), suggesting that these cells are the progeny of Lepr+ cells.

A previous report showed that periosteal cells contribute to the woven bone callus formation in the fracture healing model (Grcic et al., 2012). Because P5-iOsx/Tomato+ cells and Lepr/Tomato+ cells were also observed in the periosteum (Figures S1F and S3A), it suggests that these cells may also contribute to the callus formation. Interestingly, P5-iOsx/Tomato+ cells and Lepr/Tomato+ cells accumulated around the needle inserted into the BM for the tibia stabilization after bone fracture, suggesting that MSPC progeny also contribute to the callus formation. Interestingly, P5-iOsx/Tomato+ and Lepr/Tomato+ cells were also observed in the periosteum around the needle inserted into the BM for the tibia stabilization after bone fracture, suggesting that MSPC progeny also contribute to the callus formation. Interestingly, P5-iOsx/Tomato+ and Lepr/Tomato+ cells were also observed in the periosteum around the needle inserted into the BM for the tibia stabilization after bone fracture, suggesting that MSPC progeny also contribute to the callus formation.

The progeny of Lepr+ cells. Tomato+ cells were observed in the newly formed chondrogenic lineages, it is possible that they play important functions in regulating the proliferative and quiescent phases of HSCs. HSCs indeed proliferate markedly in the BM during development and become highly quiescent after weaning (Bowie et al., 2006). Further studies will be needed to explore the differential properties of these stromal precursors.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents

The primary antibodies used were Alexa Fluor 647-anti-VE-Cadherin (BV13) (Biorad); APC-anti-CD31/PECAM-1 (MEC13.3), APC-eFluor 780-anti-CD45 (30-F11), APC-eFluor 780-anti-Ter119 (Ter119), biotin-anti-PDGFRα (APAS), biotin-anti-PDGFRβ (APB5), and eFluor 660-anti-Ki-67 (SolA15) (all from eBioscience); anti-Lepr and anti-fatty acid binding protein 4 (FABP4) (all from R&D systems); anti-Osx (Abcam); anti-Osteocalcin (mOC1-20 and R21C-01A) and anti-DMP-1 (all from TAKARA); anti-Perilipin (D1D8) (Cell Signaling); and anti-Sox9 (Millipore). The secondary antibodies used were Alexa Fluor 647 donkey anti-goat IgG, Alexa Fluor 488 donkey anti-goat IgG, Alexa Fluor 488 donkey anti-rat IgG, and Alexa Fluor 633 goat anti-rabbit IgG (all from Molecular probes), and Streptavidin eFluor 450 (eBioscience). Alexa Fluor 488-anti-GFP (Molecular Probes) was used for enhancement of the Nes-GFP signal. Nuclei were stained with Hoechst 33342 or DAPI (4′-diamino-2-phenylindole) (all from Sigma-Aldrich). Lipid droplets were stained with BODIPY 493/503 (Molecular Probes).

Immunofluorescence Staining

Mice were perfused with 4% paraformaldehyde (PFA) for fixation, and bone tissue were further fixed with 4% PFA for 30 min at 4°C and incubated in 10%, 20%, and 30% sucrose each for 1 hr at 4°C for cryoprotection and embedded in 5% carboxymethyl cellulose (SECTION-LAB). Sections 10–30 μm thick were prepared using Kawamoto’s film method (Kawamoto and Shimizu, 2000). Images were acquired using a laser-scanning confocal microscope (SP5 AOBS, Leica), Leica LAS-AF software (Leica), and image J (Schneider et al., 2012). In the mouse fractured bone, sections were stained with Toluidine blue (Sigma-Aldrich), Lipid droplets were stained with BODIPY 493/503 (Molecular Probes).

Cell Sorting and Flow Cytometry

Cell sorting experiments were performed using an Aria Cell Sorter (BD Biosciences). Flow cytometric analyses were carried out using an LSRII flow cytometer equipped with FACS Diva 6.1 software (all BD Biosciences). Dead cells and debris were excluded by FSC, SSC, and DAPI (Sigma-Aldrich) staining profiles. Data were analyzed with FlowJo (Tree Star) or FACS Diva 6.1 software.

Statistics

Data were evaluated by unpaired Student’s t tests. Experiments were performed three times and similar results were obtained. Statistical analyses were performed with Graph Pad Prism 6. p < 0.05 was considered statistically significant.
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.devcel.2014.03.013.

ACKNOWLEDGMENTS

We thank D. Rowe, J. Butler, and S. Rafii for providing Col1(2.3)-Gfp mice. We would like to acknowledge C. Prophete, P. Ciero, L. Schiff, and A. Zahalka for technical assistance; L. Tesfa and O. Uche in the Einstein Flow Cytometry Core Facility for technical assistance of cell sorting; and P. Guo in the Analytical Imaging Facility for technical assistance in confocal microscope imaging.

S.P. is a New York Stem Cell Foundation-Druckenmiller Fellow, and M.H. is a German Research Foundation (DFG) Fellow. This work was supported by the New York Stem Cell Foundation and by R01 grants from National Institutes of Health (DE022564 to N.O., DK056246 to H.M.K., and DK056638 and HL069438 to P.S.F.).

Received: August 26, 2013
Revised: January 13, 2014
Accepted: March 18, 2014
Published: May 12, 2014

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Osterix Marks Distinct Waves of Primitive and Definitive Stromal Progenitors during Bone Marrow Development

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**Figure S1**

A. Day 1 (Tam at E13.5)  
B. Day 1 (Tam at P5)

C. 2 weeks (Tam at E13.5)
D. Graph showing the number of iOsx/Tomato cells per femur.

E. FSC vs CD31 flow cytometry for Live cells.
F. 32 weeks (Tam at P5)

G. FSC vs CD31 flow cytometry for Live cells.

H. Flow cytometry for CD45-Ter119-iOsx/Tomato (Tam at P5) for G0, S+G2/M, G1, and Non-G0.

I. Percentage of cells in G0 and Non-G0 phases for Day 1, 2 weeks, 4 weeks, and 30 weeks.
Figure S1, related to Figure 1. Osx+ cells at P5 give rise to long-lived BM stromal cells. (A-I) iOsx/Tomato mice were administered tamoxifen (Tam) at E13.5 (A, C, and D) and P5 (B, E-I), and analyzed at the indicated periods. (A and B) Z-stack confocal images of thick bone sections 1 day after Tam injection stained with Osx antibody (green). Right panels are confocal images of the boxed areas. (C) Z-stack confocal images of thick bone sections 2 weeks after Tam injection stained with osteocalcin antibody (green). Arrows: iOsx-derived Tomato+ (iOsx/Tomato+) BM stromal cells. Arrowheads: iOsx/Tomato and osteocalcin double-positive osteoblasts. (D) Quantification of the absolute number of iOsx/Tomato+ cells in the BM stromal population (CD45−Ter119−CD31−). n = 3. *P < 0.05. (E) Representative FACS plots (gated on live cells) showing percentage of CD45−Ter119−CD31− stromal cells within the iOsx/Tomato+ population 1 day after Tam injection, in the BM and bone. n = 3. (F) Z-stack confocal images of thick bone sections 32 weeks after Tam injection. Right panel is a magnified view of the boxed area. Arrows: iOsx/Tomato+ bone-lining cells. Arrowheads: iOsx/Tomato+ periosteal cells. (G) Representative FACS plots (gated on live cells) showing the percentage of CD45−Ter119−CD31− stromal cells within the iOsx/Tomato+ BM population, 15 weeks after Tam injection. n = 3. (H and I) Cell cycle analysis of iOsx/Tomato+ cells (gated on CD45−Ter119−CD31− cells) in bone (Day 1) and BM (2, 4, and 30 weeks) by FACS using anti-Ki-67 and Hoechst 33342 staining, representative plots (H) and quantification (I). n = 3. In (D, E, G, and I), data are represented as mean ± SEM. Nuclei were detected by Hoechst 33342 (blue). Scale bars represent 500 µm (F), 100 µm (A) and (B), and 50 µm (C).
Figure S2

A

B

F

15 wks (Tam at P5)  12 wks (Tam at 8 wks)

C

D

E

G

H

I

J

Day 1 Bone (Tam at P5)

CD45-Ter119-CD31- iOsx/Tomato+

Nestin-GFP−  Nestin-GFP+

1.7 ± 0.6%  6.2 ± 0.3%

2 weeks BM (Tam at E13.5)

2 weeks BM (Tam at 15 weeks)

2 weeks BM (Tam at P5)

Primary  Secondary

88.2%  89.0%

65.6 ± 3.0%

89.0%
**Figure S2, related to Figures 1 and 2. Characterization of iOsx-derived BM stromal cells.** (A-G) iOsx/Tomato mice were administered tamoxifen (Tam) at P5 (A-C, and E-G) and 8 weeks (D and E), and analyzed at the indicated periods. (A and B) Quantification of the frequency (A) and absolute number (B) of iOsx-derived Tomato+ (iOsx/Tomato+) cells in the BM stromal population (CD45−Ter119−CD31−). n = 3-4. (C and D) Z-stack confocal images of thick bone sections. Arrows: iOsx/Tomato+ bone-lining cells. (E) Percentage of cortical bone surface occupied by iOsx/Tomato+ bone-lining cells after 15 weeks chase. n = 3. (F and G) Clonal primary and secondary sphere formation assay of iOsx/Tomato+ BM stromal cells harvested 3-4 weeks after Tam injection, representative images of primary and secondary spheres (F; bright field and Tomato fluorescence on the upper and lower panels, respectively) and quantification (G). n = 4 independent experiments. (H-J) Nes-Gfp/iOsx/Tomato mice were administered Tam at E13.5 (I and J) and at P5 (H), and analyzed after 1 day (H) and 2 weeks (I and J). (H) Representative FACS plots showing the percentages of the Lepr+ cells within the iOsx/Tomato+Nes-GFP− (left) and iOsx/Tomato+ Nes-GFP+ (right) populations in bone (gated on CD45−Ter119−CD31−) detected by Lepr antibody. n = 4. (I) Confocal images of Nes-GFP and iOsx/Tomato double-positive BM stromal cells stained with Lepr antibody (white). Arrows: Nes-GFP, iOsx/Tomato, and Lepr triple-positive cells. (J) Representative FACS plots showing the percentages of Nes-GFP and Lepr double-positive cells, and the expression of PDGFRα and PDGFRβ, within the iOsx/Tomato+ (CD45−Ter119−CD31−) BM stromal population. n = 3. Blue and red lines represent isotype controls and antibodies against the markers indicated, respectively. *P < 0.05, **P < 0.01, NS: not significant. In (A, B, E, G, H and J), data are represented as mean ± SEM. Scale bars represent 300 μm (C) and (D), 200 μm (F), and 20 μm (I).
Figure S3

A) CD45-Ter119-CD31-
Lepr-cre/Tomato
Nestin-GFP

Percentage of cells
0 10^2 10^3 10^4 10^5
0 20 40 60 80 100

PDGFRα
PDGFRβ

B) C

D) E

F) CD45-Ter119-CD31-
Lepr-cre/Tomato+

Percentage of cells
0 100 200 300 400

G) Nestin-GFP

H) 15 weeks iOsx/Tomato mice (2 days after Tam injection)

BM

Bone

iOsx/Tomato

I)

Lepr (relative mRNA)

Lepr-cre/Lepr-cre
Lepr-cre/Tomato

Osteoblasts

BM stromal cells

J)

Osterix (relative mRNA)

Lepr-cre/Lepr-cre
Lepr-cre/Tomato

BM stromal cells

3.0%
95.5%

92.4%

92.4%

0.1
0.2
0.3
0.4
0.5

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**Figure S3, related to Figure 3. Characterization of Lepr-cre-derived BM stromal cells.** (A) Representative FACS plots (gated on live CD45−Ter119−CD31− cells) showing the expression of Nes-GFP, PDGFRα, and PDGFRβ in the Lepr-cre-derived Tomato+ (Lepr/Tomato+) BM population in 8-week-old Nes-Gfp/Lepr-cre/Tomato mice. Blue and red lines represent isotype controls and antibodies against the markers indicated, respectively. (B-D) Differentiated phenotypes of clonal Lepr/Tomato+ BM stromal cells shown by Alizarin Red S: osteoblasts (B), Alcian Blue: chondrocytes (C), and lipid droplets and staining with FABP4 antibody: adipocytes (D). (E) Confocal images of bone tissue in primary spongiosa from 1-week-old Nes-Gfp/Lepr-cre/Tomato mice stained with osterix antibody (white). Arrows: Nes-GFP, Lepr/Tomato, and Osx triple-positive cells. (F) Representative FACS plot showing the percentage of Nes-GFP+ cells in the Lepr-cre/Tomato+ (CD45−Ter119−CD31−) BM population from 1-week-old Nes-Gfp/Lepr-cre/Tomato mice. Blue and red lines represent WT control and Nes-GFP, respectively. (G) Z-stack confocal images of thick bone sections in 15-week-old Lepr-cre/Tomato mice. Right panel shows magnified view of boxed area. Arrows: Trabecular bone. (H) Z-stack confocal image of thick bone sections from 15-week-old iOsx/Tomato mice 2 days after tamoxifen (Tam)-injection. iOsx-derived Tomato+ (iOsx/Tomato)+ cells were observed as osteoblasts but not as BM stromal cells. Arrows: iOsx/Tomato+ osteoblasts. (I and J) iOsx/Tomato+ osteoblasts (gated on live CD45−Ter119−CD31− cells) were sorted from digested cortical bone tissue of 15-week-old iOsx/Tomato mice 2 days after Tam injection. Lepr/Tomato-positive and -negative BM stromal cells (gated on live CD45−Ter119−CD31− cells) were sorted from digested BM cells in 15-week-old Lepr-cre/Tomato mice. Expression levels of Lepr (I) and Osterix (J) mRNA were measured by quantitative real-time PCR. n = 3. **P < 0.01, ***P < 0.001. In (I and J), data are represented as mean ± SEM. Nuclei were detected by Hoechst 33342 or DAPI (blue). Scale bars represent 2000 µm (B), 500 µm (G), 100 µm (C) and (H), and 30 µm (D) and (E).
**Figure S4, related to Figure 4. Lepr-cre-derived BM stromal cells contribute to adipocytes and chondrocytes.** (A) Z-stack confocal images of thick bone sections at the indicated time points after irradiation, stained with VE-cadherin (VE-Cad), PECAM-1 (white), and Perilipin antibodies (green). (B and C) Lepr+ BM cell populations from Lepr-cre/iDTR and control (iDTR) mice at 3 days after administration of diphtheria toxin (DT) were detected by Lepr antibody, representative FACS plot (gated on live CD45−Ter119−CD31− cells) (B) and quantification (C). n = 3. (D and E) Lepr-cre/iDTR and iDTR mice were irradiated 1 day after administration of DT, and analyzed 6 days later. Z-stack confocal images of thick bone sections stained with VE-Cad, PECAM-1 (white) and Perilipin antibodies (green) (D) and quantification (number of adipocytes in an area of 2 mm² under the growth plate) (E). n = 3. (F) Z-stack confocal images of thick bone sections 8 days post bone fracture in 15-week-old Lepr-cre/Tomato mice stained with Lepr antibody (white). The numbered squares indicate the area of the lower panels. *: Fracture callus. Nuclei were detected by Hoechst 33342 (blue). *P < 0.05, **P < 0.01, ***P < 0.001. In (C and E), data are represented as mean ± SEM. Scale bars represent 500 μm (A) and (F), and 200 μm (D).
Supplemental Experimental Procedures

**CFU-F assay**

1-3 x 10³ sorted cells were seeded per well in a 12-well adherent tissue culture plate containing phenol-red free α-MEM (Gibco) supplemented with 20% FBS (Hyclone), 10% MesenCult stimulatory supplement (StemCell Technologies) and 0.5% penicillin-streptomycin. One-half of the media was replaced after 7 days. Cells were stained with Giemsa staining solution (EMD Chemicals) and adherent colonies were counted at culture day 14.

**In vitro cell differentiation**

For osteogenic, adipogenic and chondrogenic differentiation, clonally expanded P5-iOsx/Tomato⁺ or Lepr/Tomato⁺ BM stromal cells isolated from CFU-F cultures, were treated with StemXVivo osteogenic, adipogenic or chondrogenic differentiation media, according to manufacturer’s instructions (R&D Systems). Cultures were maintained with 5% CO₂ in a water-jacketed incubator at 37°C during 4-6 weeks and half-medium changes were performed bi-weekly. Osteogenic differentiation indicated by mineralization of extracellular matrix and calcium deposits was revealed by Alizarin Red S staining. Cells were washed twice with PBS and fixed with 4% paraformaldehyde (PFA) for 30 min. After rinsing in distilled water, cells were stained with 40 mM Alizarin Red S (Sigma-Aldrich) solution at pH 4.2, rinsed in distilled water, and washed in Tris-buffered saline for 15 min to remove nonspecific staining. Adipocytes were identified by the characteristic production of lipid droplets and staining with anti-FABP4 antibody. Chondrocytic differentiation was assessed by Alcian Blue staining of the
mucopolysaccharides. Cells were washed twice with PBS and fixed with 10% formalin for 60 min, rinsed in distilled water, and stained with 1% Alcian Blue 8GX solution in 3% acetic acid (Sigma-Aldrich) overnight. To remove nonspecific staining, cells were incubated with a 6:4 dilution of ethanol:acetic acid for 20 min and finally washed with PBS.

**Spheroid formation assay**

For spheroid formation assay, clonally expanded P5-iOsx/Tomato+ BM stromal cells isolated from CFU-F cultures were transferred to non-adherent 35 mm plates (10,000 cells/plate) with spheroid-forming media (Mendez-Ferrer et al., 2010; Pinho et al., 2013) [1:2 ratio of DMEM F12 (Gibco) and Human Endothelial Medium (Gibco) supplemented with 3.75% Chicken Extract (US Biological), 0.1mM β-ME (Invitrogen), 1% Non-essential aminoacids (Gibco), 1% Pen-strep (Gibco), 1% N2 (Gibco), 2% B27 (Gibco), 20 ng/mL human bFGF (R&D Systems), 20 ng/mL mouse PDGF-AA (Peprotech), 20 ng/mL mouse oncostatin M (R&D Systems), 20 ng/mL mouse IGF-1 (Peprotech), 20 ng/mL mouse EGF (Peprotech)]. After 7 days, the primary spheroid-forming efficiency was determined. Spheres were dissociated with Accutase (Invitrogen) and re-seeded in fresh-spheroid-forming media for the formation of secondary spheres. After 7 days, the secondary spheroid-forming efficiency was determined.

**Induction of Cre-mediated recombination and iDTR-mediated cell depletion**

E13.5 pregnant mice were injected with 2 mg/40 g tamoxifen (Tam) (Sigma-Aldrich) i.p. and co-injected with 1 mg/40 g progesterone (Sigma-Aldrich) to minimize
the rate of abortions due to Tam injection (Nakamura et al., 2006). P5 mice were injected with 2 mg/30 g Tam by intragastric injection. 8-15-week-old mice were injected with 4 mg/30 g Tam i.p. 2 times every other day. For diphtheria toxin (DT)-mediated cell depletion, 100 ug/kg of DT (Sigma) was injected intraperitoneally.

**Preparation of BM and bone cell suspension**

Both sides of mouse femur were cut, and BM was gently flushed in L-15 FACS buffer (Mendez-Ferrer et al., 2010). Bone tissues including both sides were crushed with mortar. BM or bone fragments were digested with 0.1% collagenase IV (Sigma-Aldrich) and 0.2% Dispase (Gibco) in HBSS (Gibco) for 30 min at 37°C.

**Cell cycle analysis**

Cell cycle analysis was performed as described previously (Kunisaki et al., 2013; Wilson et al., 2008). In brief, bone marrow cells were stained with surface markers, fixed in 2% PFA in PBS, washed, permeabilized with 0.1% Triton X-100 in PBS, and stained with anti-Ki67 antibody and Hoechst 33342. After washing, cells were analyzed in a LSRII Flow Cytometer (Becton Dickinson).

**Bone fracture and irradiation injury**

Fracture wound healing was studied in 15-week-old Lepr-cre/Tomato and 32-week-old P5 pulsed iOsx/Tomato mice using a semi-stabilized tibiae fracture model (Maes et al., 2006). Mice were analyzed 8 days after bone fracture. For radiation injury,
8-week-old Lepr-cre/Tomato and 15-week-old P5 pulsed iOsx/Tomato mice were irradiated with 6 Gy and subsequently analyzed 6 days after.

**RNA isolation and quantitative real-time PCR**

Sorted cells were collected in lysis buffer and RNA isolation was performed using the Dynabeads® mRNA DIRECT™ Micro Kit (Invitrogen). Reverse transcription was performed using the RNA to cDNA EcoDryTM Premix system (Clontech), following the manufacturer’s recommendations. Quantitative real-time PCR was performed as previously described (Mendez-Ferrer et al., 2010). The relative mRNA abundance was calculated using the ΔCt method. Gene expression data was normalized to Gapdh. The sequences of primers for each gene were as follows: Lepr, 5’-TCAGAATTTTGGGTGAAATA-3’ (forward) and 5’-GTCCAGGTGAGGAGCAAGAG-3’ (reverse); Osterix, 5’-ATGGCGTCCTCTCTGCTTGA-3’ (forward) and 5’-GAAGGGTGGGTAGTCATTTG-3’ (reverse); Gapdh, 5’-TGTGTCCGTCGTGGATCTGA-3’ (forward) and 5’-CC TGCTTCACCACCTTCTTGAGTA-3’ (reverse).
Supplemental References

