Bone marrow and periosteal skeletal stem/progenitor cells make distinct contributions to bone maintenance and repair

Graphical abstract

**Authors**
Elise C. Jeffery, Terry L.A. Mann, Jade A. Pool, Zhiyu Zhao, Sean J. Morrison

**Correspondence**
sean.morrison@utsouthwestern.edu

**In brief**
In adult mice, different skeletal stem/progenitor cells (SSCs) are responsible for the repair of distinct bone injuries. LepR\(^+\)Adiponectin\(^+\) bone marrow SSCs are responsible for steady-state osteogenesis and drill injury repair while Gli1\(^+\) periosteal SSCs are primarily responsible for bicortical fracture repair and bone marrow stromal cell regeneration at fracture sites.

**Highlights**
- *Adiponectin-cre* marks LepR\(^+\) bone marrow SSCs, and *Gli1\(^{creERT2}\)* marks periosteal SSCs
- LepR\(^+\)Adipoq\(^+\) bone marrow SSCs are responsible for adult steady-state osteogenesis
- LepR\(^+\)Adipoq\(^+\) bone marrow SSCs are responsible for the repair of drill-hole injuries
- Gli1\(^+\) periosteal SSCs are primarily responsible for the repair of bicortical fractures
Bone marrow and periosteal skeletal stem/progenitor cells make distinct contributions to bone maintenance and repair

Elise C. Jeffery, Terry L.A. Mann, Jade A. Pool, Zhiyu Zhao, and Sean J. Morrison

INTRODUCTION

Skeletal stem/progenitor cells (SSCs) that are capable of contributing to bone repair are present in the peristeum on the outside surface of bones (Duchamp de Lageneste et al., 2018; Debnath et al., 2018; Ortinau et al., 2019; Matthews et al., 2021; Zhang et al., 2005), as well as inside the bone marrow (Chan et al., 2015; Zhou et al., 2014; Matsushita et al., 2020; Bianco and Robey, 2015; Friedenstein et al., 1970). However, the relative contributions of these SSC populations to bone repair have been debated, in part because few markers have been available to distinguish these cell populations. Much of the fate mapping that has been performed was done on early postnatal or juvenile mice when different cells are responsible for the formation and repair of bone as compared to adulthood (Ono et al., 2014a, 2014b; Mizoguchi et al., 2014; Leucht et al., 2008). Moreover, few studies have performed quantitative, side-by-side comparisons of the contributions of different SSC populations to bone repair, making it difficult to compare results among studies.

Within adult bone marrow, leptin receptor-expressing (LepR⁺) SSCs are the major source of new osteoblasts for bone maintenance and fracture repair (Zhou et al., 2014). These cells, or subsets of these cells, also express PDGFRα (Morikawa et al., 2009), CD146 (Sacchetti et al., 2007), CDS1 (Chan et al., 2015; Zhou et al., 2014), Adiponectin (Zhong et al., 2020), Osteolectin (Yue et al., 2016; Shen et al., 2021), Hoxa11 in the zeugopod (Pineault et al., 2019), stem cell factor (SCF) (Ding et al., 2012) and Cxcl12 (Matsushita et al., 2020; Omatsu et al., 2010; Ding and Morrison, 2013). Bone marrow LepR⁺ SSCs arise perinatally from perichondrial cells that express Osterix (Mizoguchi et al., 2014; Maes et al., 2010), Aggrecan (Ono et al., 2014a; Shu et al., 2021), Hoxa11 (Pineault et al., 2019), and Collagen 2 (Ono et al., 2014a, 2014b; Shu et al., 2021). Bone marrow LepR⁺ cells are heterogeneous (Tikhonova et al., 2019; Baryawno et al., 2019; Baccin et al., 2020), including periarteriolar Osteolectin⁺ cells that are fated to form osteoblasts (Shen et al., 2021) and perisinusoidal Osteolectin negative cells that are normally fated to form adipocytes (Zhong et al., 2020; Zhou et al., 2017) but form bone after bone injuries (Matsushita et al., 2020). Most of the markers that have been used to identify and fate map LepR⁺ SSCs in the bone marrow have not been assessed in the peristeum, creating uncertainty about whether these markers distinguish bone marrow from peristeal SSCs.

In fetal and postnatal mice prior to one month of age, Gli1creERT2 marks SSCs in the perichondrium that promote bone growth and repair as well as giving rise to LepR⁺ stromal cells in the bone marrow (Shi et al., 2017). After one month of age, Gli1creERT2 expression is restricted to the metaphysis and declines quickly in young adult mice (Shi et al., 2017). Gli1creERT2 also labels SSCs in calvarial sutures that contribute to bone growth and repair (Zhao et al., 2015). Gli1 is expressed by a
subset of cells in the periosteum of adult bones, but it has not yet been tested whether these cells have SSC activity (Shi et al., 2017; Xu et al., 2022). These studies raise the question of whether Gli1creERT2 labels cells that contribute to the repair of adult bones beyond the calvarium, which is unusual as it forms largely via intramembranous ossification.

The periosteum of adult bones includes an inner cambial layer adjacent to the bone surface and an outer fibrous layer (Chang and Knothe Tate, 2012). There is evidence for SSCs in both layers (Duchamp de Lageneste et al., 2018; Ortinau et al., 2019; Xu et al., 2022; Ito et al., 2001). While both periosteal SSCs and bone marrow SSCs have been reported to contribute osteoblasts to fracture repair (Debnath et al., 2018; Ortinau et al., 2019; Matthews et al., 2021; Zhou et al., 2020; Colnot, 2009), transplantation of marked periosteal cells suggested that chondrocytes in the cartilage callus around bicortical fractures arise primarily from periosteal cells (Colnot, 2009). Adult periosteal SSCs express PDGFRα (Duchamp de Lageneste et al., 2018; Matthews et al., 2021; Xu et al., 2022), z-smooth muscle actin (Acta2) (Ortinau et al., 2019; Matthews et al., 2016, 2021), and Pmr1 (Duchamp de Lageneste et al., 2018; Kawanami et al., 2009; Murao et al., 2013). By flow cytometry, they are enriched among Sca1+CD51+ cells and CD90+CD105- cells (Matthews et al., 2021; Mo et al., 2021). Nonetheless, most of these markers are also expressed by LepR+ bone marrow SSCs (Ortinau et al., 2019; Matthews et al., 2021; Zhou et al., 2014; Logan et al., 2002; Greenbaum et al., 2013), making it difficult to distinguish the contributions of periosteal cells and bone marrow LepR+ cells to bone repair by fate marking.

Bone injuries can heal through intramembranous ossification, in which SSCs differentiate directly to bone, or through endochondral ossification, in which SSCs form a cartilage intermediate and then bone (Serowoky et al., 2020). Drill-hole injuries are small, stabilized fractures (Matsushita et al., 2020; Leucht et al., 2009) that heal through intramembranous ossification (Colnot, 2009; Serowoky et al., 2020; Thompson et al., 2002). Non-stabilized or partially stabilized bicortical fractures heal primarily through endochondral ossification with the formation of a cartilage callus followed by osteogenesis (Colnot, 2009; Serowoky et al., 2020; Kuwahara et al., 2019). The observation that different bone injuries induce different modes of repair raises the question of whether they are healed by different SSCs.

We performed lineage tracing with 11 Cre alleles to identify markers that distinguish periosteal from bone marrow SSCs. Most Cre alleles labeled both types of SSCs. However, we found that Gli1creERT2 preferentially labeled periosteal SSCs in the diaphysis of adult long bones while Adiponectin-cre and Adiponectin-creERT specifically labeled LepR+ bone marrow SSCs. Lepcrcre labeled bone marrow SSCs but also recombined in a minority of periosteal SSCs. These markers allowed us to compare the contributions of periosteal versus bone marrow SSCs to the repair of drill-hole injuries and bicortical fractures.

RESULTS

Identification of adult bone marrow SSCs
To identify Cre alleles that most effectively mark adult bone marrow SSCs, we performed side-by-side fate mapping with several Cre alleles that have been reported to mark SSCs, including Nestin-creERT2 (Méndez-Ferrer et al., 2010), Grem1-creERT2 (Worthley et al., 2015), Osterix-creERT2 (Mizoguchi et al., 2014; Maes et al., 2010; Park et al., 2012), Gli1creERT2 (Shi et al., 2017; Zhao et al., 2015), Lepcrcre (Zhou et al., 2014), Adiponectin-cre (Zhong et al., 2020; Mukohira et al., 2019; Zhang et al., 2021), and Adiponectin-creERT2 (Zhong et al., 2020; Zhou et al., 2017). In each genetic background, we quantitated the contributions of labeled cells to all bone marrow stromal cells, LepR+ bone marrow stromal cells, Col1(2.3)-GFP+ osteoblasts using flow cytotmetry (Kalajzic et al., 2002), and colony-forming units-fibroblast (CFU-F) at 8 to 24 weeks of age. In CreER-expressing mice, we treated with tamoxifen at 8 weeks of age and then quantitated the contributions of labeled cells at 3 days to 8 weeks after tamoxifen treatment. Consistent with prior studies (Zhou et al., 2014; Ono et al., 2014b; Mizoguchi et al., 2014; Worthley et al., 2015; Green et al., 2021), in adult Nestin-creERT2; tdTomato mice less than 3% of bone marrow stromal cells, LepR+ cells (identified based on staining with an anti-LepR polyclonal antibody), osteoblasts, and CFU-F were labeled in the diaphysis and metaphysis (Figure S1A). Grem1-creERT2; tdTomato mice exhibited transient labeling of 7% of LepR+ cells in the metaphysis at 2 weeks after tamoxifen treatment, but otherwise less than 1% of bone marrow stromal cells, LepR+ cells, osteoblasts, and CFU-F in the diaphysis and metaphysis were Tomato+ (Figure S1B). This is consistent with the observation that Grem1-creERT2-expressing cells are present near the growth plate and are depleted as mice enter adulthood (Worthley et al., 2015). Nestin-creERT and Grem1-creERT2, therefore, did not label significant numbers of SSCs in adult bone marrow.

Adult Osterix-creERT2; tdTomato mice exhibited labeling in less than 4% of bone marrow stromal cells, 5%–10% of LepR+ stromal cells in the bone marrow metaphysis, 5%–15% of CFU-F in the metaphysis and diaphysis, and 60%–100% of osteoblasts, though the percentage of labeled osteoblasts declined over time (Figure S1C). These data are consistent with Osterix-creERT2 recombination in osteoblasts (Park et al., 2012) and suggest Osterix-creERT2 recombine in a minority of SSCs in adult bone marrow.

Gli1creERT2; tdTomato mice exhibited recombination in less than 1% of bone marrow stromal cells, LepR+ cells, or CFU-F in the adult bone marrow diaphysis (Figure S1D). A higher level of recombination was observed transiently in the bone marrow metaphysis, where in the first few weeks after tamoxifen treatment up to 10% of bone marrow stromal cells, 13% of LepR+ cells, 26% of osteoblasts, and 12% of CFU-F were Tomato+ (Figure S1D). However, the amount of labeling in the metaphysis declined over time. These results are consistent with the observation that Gli1creERT2 labels a subset of SSCs in the metaphysis but not in the diaphysis, and that Gli1creERT2 expressing cells in the metaphysis are rapidly depleted in young-adult mice (Shi et al., 2017).

In contrast to these markers that labeled few adult bone marrow SSCs, Lepcrcre and Adiponectin-cre labeled most of the CFU-F in the metaphysis and diaphysis at 8 to 24 weeks of age (Figures 1A and 1B). Adiponectin-creERT also labeled 55%–65% of CFU-F at 4 to 12 weeks after tamoxifen treatment in the metaphysis and diaphysis (Figure 1C). Lepcrcre and Adiponectin-cre each labeled less than 13% of all bone marrow...
stromal cells but 65%–90% of LepR+ stromal cells in the metaphysis and diaphysis (Figures 1A and 1B). Consistent with prior studies (Zhou et al., 2014; Mukohira et al., 2019), they also labeled an increasing percentage of endosteal osteoblasts over time in adult mice, including 15%–40% of osteoblasts in the metaphysis and diaphysis of 24-week-old mice (Figures 1A and 1B). Adiponectin-creERT labeled less than 5% of all bone marrow stromal cells but 30%–40% of LepR+ cells and 15%–30% of osteoblasts in the metaphysis and diaphysis at 4 and 12 weeks after tamoxifen treatment.

Consistent with these results, single-cell RNA sequencing has shown that LepR and Adiponectin are expressed by the same cells in adult bone marrow (Zhong et al., 2020; Tikhonova et al., 2019; Baryawno et al., 2019). Nearly all (92%–98%) Tomato+ stromal LepR and Adiponectin expression strongly overlap in the bone marrow.

LepR is expressed by a subset of periosteal SSCs, but Adiponectin is not

To begin to characterize SSCs in the periosteum, we analyzed by flow cytometry the expression of Sca1 and CD51, markers associated with periosteal SSCs (Matthews et al., 2021; Mo et al., 2021). Within the periosteum of 8-week-old mice, Sca1+CD51+ cells were enriched for CFU-F (12 ± 4.6% of cells in the bone marrow of 8-week-old LepRcre; tdTomato (Figure 1D), Adiponectin-cre; tdTomato (Figure 1E), and Adiponectin-creERT; tdTomato (Figure 1F) mice). The numbers in each panel reflect n = 3–6 mice from 1–3 independent experiments. All data represent mean ± SD.
Sca1^+CD51^+ cells formed fibroblast colonies, while Sca1^+CD51^+ cells had limited CFU-F activity (2.5 ± 1.8% formed colonies) and Sca1^+CD51^+ cells had no CFU-F activity (Figures 2A and 2B). The Sca1^+CD51^+ cells were present in both the cambial and fibrous layers of the periosteum (Figure S2B).

To test if markers that identified SSCs in bone marrow were also expressed by SSCs in periostem we assessed recombination by Leprcre, Adiponectin-cre, and Adiponectin-creERT in periosteal cells from 10– to 16-week-old mice by flow cytometry. We observed recombination by Leprcre in 6.7 ± 2.0% of non-hematopoietic periosteal cells (Figures 2C and 2F) but did not detect recombination by Adiponectin-cre (Figures 2D and 2F) or Adiponectin-creERT (Figure 2F). Leprcre also recombined in 16 ± 8.7% of periosteal CFU-F (Figures 2G) and 11 ± 4.8% of periosteal Sca1^+CD51^+ cells (Figure 2H). We did not detect recombination in any of these periosteal cell populations in Adiponectin-cre or Adiponectin-creERT mice (Figures 2F–2H). Thus, Leprcre recombined in a minority of SSCs in adult periostem but Adiponectin-cre and Adiponectin-creERT did not.
Identification of adult periosteal SSCs

We performed fate mapping with several Cre alleles that could potentially mark periosteal SSCs including CathepsinK (Ctsk)cre (Debnath et al., 2018), Mx1-Cre (Ortinau et al., 2019; Park et al., 2012), Periostin-cre (Duchamp de Lageneste et al., 2018; Horiuchi et al., 1999), and αSMA-creERT2 (Matthews et al., 2016, 2021). In Ctskcre; tdTomato mice, 30 ± 8.3% of Stromal cells in 8–10-week-old adult periosteum and 1%–3% of Bone marrow Stromal cells were Tomato+ (Figure S2C). However, 49%–52% of Bone marrow LeprR+ cells and nearly all Periosteal and Bone marrow CFU-F were Tomato+ (Figures S2C and S2G). Ctskcre; tdTomato thus appeared to recombining broadly in SSCs from adult periosteum and Bone marrow.

We treated 8-week-old Mx1-Cre; tdTomato mice with polyinosine-polycytidylate (pIpC) to induce recombination. One to two weeks later, 10%–20% of Stromal cells in the Bone marrow and periosteum (Figure S2D), 41%–45% of LeprR+ cells in the Bone marrow, 56%–64% of CFU-F in Bone marrow, and 26% of CFU-F in periosteum were Tomato+ (Figure S2D). Mx1-Cre thus recombined in both Bone marrow and periosteal SSCs, consistent with prior studies (Ortinau et al., 2019; Park et al., 2012).

Periostin is expressed in the periosteum but not in the Bone marrow, and its expression in periosteal cells increases after fracture (Duchamp de Lageneste et al., 2018; Horiuchi et al., 1999). In 8– to 10-week-old Periostin-cre; tdTomato mice, less than 1% of Stromal cells, LeprR+ Bone marrow cells, and CFU-F were Tomato+ in the periosteum and the Bone marrow (Figure S2E). Even after drill injury in the tibia diaphysis, we still only observed rare Tomato+ Stromal cells or Osteoblasts (data not shown). Periostin-cre, therefore, was not an effective marker of Adult periosteal SSCs despite Periostin expression by these cells.

αSMA-creERT2 is expressed by a subset of Adult periosteal cells that are enriched for CFU-F activity, and the number of positive cells increases after fracture (Ortinau et al., 2019; Matthews et al., 2021). We treated 8– to 10-week-old αSMA-creERT2; tdTomato mice (Wendling et al., 2009) with tamoxifen and observed that approximately 10% of Stromal cells and CFU-F in the periosteum were Tomato+ 3–6 days after tamoxifen treatment (Figure S2F). In the Bone marrow, less than 3% of Stromal cells, LeprR+ cells, and CFU-F were Tomato+ in the diaphysis and metaphysis (Figure S2F). Under steady-state conditions, most of the Tomato+ cells in the Bone marrow of αSMA-creERT2; tdTomato mice were periarteriolar Stromal cells, consistent with smooth Muscle cells. Fewer than 5% of the Tomato+ cells in the Bone marrow stained positively for LeprR by flow cytometry.

Although few cells in the periosteum or Bone marrow recombined with αSMA-creERT2 under steady-state conditions (Figure S2F), we tested whether administration of tamoxifen after injury would result in increased recombination, as shown previously with a different αSMA-creERT2 allele (Matthews et al., 2016, 2021). We performed drill injuries or bicortical fractures in αSMA-creERT2; tdTomato mice then treated with tamoxifen for up to 24 h after the injury (Figures S3A and S3B). We observed a substantial increase in the number of Tomato+ cells near the injury sites as compared to uninjured bones at 7 to 10 days after the injury (compare Figures S3D and S3G to Figure S2F). However, these Tomato+ cells were present in both the periosteum and the Bone marrow near the injury sites. The Tomato+ cells in the Bone marrow could not have derived from Gli1+ periosteal cells as these cells do not give rise to cells within the Bone marrow 10 days after drill injuries (Figure S3C). This suggests that Acta2 (which encodes αSMA) was induced in Bone marrow Stromal cells and Periosteal cells after the Bone injuries. To assess this, we analyzed published single-cell RNA sequencing data that compared the gene expression profiles of Tomato+ Stromal cells from fractured and unfractured bones/Bone marrow of LeprRcre; tdTomato mice (Mo et al., 2021). The data included one cell cluster (#4) of periosteal cells and two clusters of LeprR+ Bone marrow Stromal cells (#7 and #10) (Figures S4A-D) that exhibited a strong induction of Acta2 after fractures as compared to unfractured or irradiated bones (Figure S4E). These data are consistent with our lineage tracing in suggesting that αSMA (Acta2) is induced in both LeprR+ Bone marrow Stromal cells and Periosteal cells after Bone injuries. αSMA-creERT2; tdTomato+ cells gave rise to Osteoblasts after drill injuries (Figure S3E) and Osteoblasts and Chondrocytes after Bicortical fractures (Figures S3H and S3I), but this CreER allele did not distinguish between periosteal and Bone marrow SSCs after Bone injuries.

When we treated 8-week-old Gli1CreERT2; tdTomato mice with tamoxifen, we observed Tomato expression in the periosteum but not in the Bone marrow diaphysis (Figures 2E and 2I). In the periosteum, 19 ± 7.5% of all cells that were not hematopoietic or endothelial cells (Figure 2I), 44 ± 13% of CFU-F (Figure 2J), and 36 ± 12% of Sca1+CD51+ cells (Figure 2K) were Tomato+ 1–2 weeks after tamoxifen treatment. Gli1CreERT2; tdTomato+ periosteal cells were present in both the cambial and fibrous layers of the periosteum (Figure S4F). In contrast, within the Bone marrow diaphysis, less than 2% of Stromal cells and CFU-F were Tomato+ (Figures 2I and 2J). Consistent with Figure S1D, we observed modestly increased labeling in the Bone marrow metaphysis, where 3.6 ± 3.1% of Stromal cells (Figure 2I) and 21 ± 11% of CFU-F (Figure 2J) were Tomato+. These data suggest that in the diaphysis of Adult bones, Gli1CreERT2 preferentially labeled periosteal SSCs.

To determine whether there is any overlap between Gli1+ periosteal cells and LeprR+ periosteal cells, we stained sections of bones from LeprRcre; tdTomato mice with an antibody against Gli1. Some Tomato+ periosteal cells stained positively for Gli1 (Figures S4I and S4J), suggesting substantial overlap between the Gli1+ and LeprR+ cells in the periosteum.

To identify the 50%–60% of Sca1+CD51+ cells in the periosteum that were Tomato negative in Gli1CreERT2; tdTomato mice (Figures 2J and 2K), we treated them with tamoxifen and, 3 days later, isolated periosteal Tomato+Sca1+CD51+ and Tomato negative Sca1+CD51+ cells. We then measured the expression of Gli1 in these two cell populations by quantitative real-time-PCR. Tomato+Sca1+CD51+ cells expressed the highest levels of Gli1 (Figure S4H). Tomato negative Sca1+CD51+ periosteal cells expressed lower levels, but nonetheless had levels of Gli1 significantly higher than LeprR+ Bone marrow Stromal cells (Figure S4H). This suggests incomplete recombination by Gli1CreERT2 in Gli1+ periosteal cells, with recombination occurring preferentially in the cells with the highest levels of Gli1.
Adult cortical osteocytes arise primarily from bone marrow SSCs under steady-state conditions

To assess the contribution of Gli1creERT2-expressing periosteal SSCs to the production of osteocytes under steady-state conditions, we treated 8-week-old Gli1creERT2; tdTomato mice with tamoxifen and then analyzed cortical bone in the femur diaphysis at 16 weeks of age. Only 2.3 ± 2.2% of osteocytes in the distal femur and 2.2 ± 1.8% of osteocytes in the proximal femur were Tomato+, and these were consistently observed near the endosteme, not near the periosteum (Figures 2L and 2M). This suggests that there are rare Tomato+ osteocytes that arise from rare Gli1creERT2-expressing cells in the bone marrow, where Gli1creERT2 labeled up to 6% of osteoblasts and 1% of bone marrow CFU-F in the femur diaphysis (Figure 1D). A similar analysis performed at 20 weeks of age found that less than 2% of osteocytes in the distal and proximal femurs were Tomato+ (Figure 2N). We thus detected little contribution of periosteal cells to the production of osteocytes under steady state conditions in young adult femurs.

We also assessed the contributions of Adiponectin-cre- and Adiponectin-creERT-expressing bone marrow SSCs to steady-state osteogenesis in the cortical femur diaphysis. We found that in young-adult mice, new osteocytes overwhelmingly arose at the distal end of the femur, near the knee, as compared to the proximal end of the femur, near the hip (Figure 2M). While few osteocytes arose from Gli1+ periosteal cells, 19 ± 8.2% of osteocytes at the distal end of the femur, near the knee, as compared to the proximal end of the femur, near the hip (Figure 2M). While few osteocytes arose from Gli1+ periosteal cells, 19 ± 8.2% of osteocytes were Tomato+ in the distal femur of 16-week-old Adiponectin-cre; tdTomato mice, and 16 ± 3.0% of osteocytes were Tomato+ in the distal femur of Adiponectin-creERT; tdTomato mice 16 weeks of age (8 weeks after tamoxifen treatment) (Figures 2L and 2M). At 24 weeks of age, 27 ± 7.8% of osteocytes were Tomato+ in the distal femur of Adiponectin-cre; tdTomato mice (Figure 2O). These Tomato+ osteocytes were consistently observed near the endosteme, not the periosteum (Figure 2L). These data suggest that most new cortical bone osteocytes in the femur diaphysis of young-adult mice arise from bone marrow SSCs.

Drill injuries are healed primarily by bone marrow SSCs

To characterize the contributions of bone marrow and periosteal SSCs to the repair of bone injuries, we used Gli1CreERT2 as a marker of periosteal SSCs and Adiponectin-cre as a marker of bone marrow SSCs in adult femur and tibia diaphysis. While Adiponectin-creERT also marked bone marrow SSCs, the recombination efficiency of Adiponectin-cre was higher (Figure 1C). We thus compared the contributions of bone marrow and periosteal SSCs to the repair of drill injuries in the tibia diaphysis of 8- to 12-week-old Adiponectin-cre; tdTomato and Gli1CreERT2; tdTomato mice (Figure 3A). In this injury model, the periosteum was left intact, and a small dental drill with 0.8 mm diameter was used to create a hole all the way through the bone cortex on one side of the tibia diaphysis, exposing the bone marrow. In non-injured contralateral control tibia from these mice, Adiponectin-cre-labeled bone marrow stromal cells and Gli1CreERT2-labeled periosteal stromal cells were relatively quiescent, with fewer than 4% of cells incorporating a 2-day pulse of 5'-ethyl-2'-deoxyuridine (EdU) (Figures 3B and 3C). However, in the tibia with the drill injury, 7.7 ± 3.6% of Adiponectin-cre-labeled bone marrow stromal cells and 20 ± 9.0% of Gli1CreERT2-labeled periosteal cells in the diaphysis incorporated a 2-day pulse of EdU, initiated 24 h after the drill injury (Figures 3B and 3C). Periosteal SSCs and bone marrow SSCs near the injury site thus became more proliferative after the injury.

We compared the contributions of periosteal and bone marrow SSCs to the repair of drill injuries in the tibia diaphysis of 8- to 12-week-old Adiponectin-cre; tdTomato; Col1(2.3)-GFP mice, LepRcre; tdTomato; Col1(2.3)-GFP mice, and Adiponectin-creERT2; tdTomato; Col1(2.3)-GFP mice (treated with tamoxifen at 8 weeks of age and injured 3–7 days later). The injuries healed over a period of 5 weeks (Figure 3A) without the generation of significant numbers of chondrocytes at the injury site (Figures SSA–SSE) (Matsushita et al., 2020; Leucht et al., 2008; Colnot, 2009; Serowowsky et al., 2020; Thompson et al., 2002). In each background, we analyzed the percentage of Col1(2.3)-GFP+ osteoblasts that were Tomato+ at 10, 22, and 35 days after injury. Adiponectin-cre and LepRcre each recombined in 50%–80% of Col1(2.3)-GFP+ osteoblasts at the injury site, while Gli1CreERT2 recombined in only about 10% of osteoblasts at the injury site (Figures 3D–3J). These results mirrored what we observed among osteocytes in the regenerated cortical bone 35 days after the injury; Adiponectin-cre and LepRcre each labeled 25%–45% of osteocytes, while Gli1CreERT2 labeled only about 5% of osteocytes (Figures 3K and S6A–S6C). We also assessed the contributions of bone marrow SSCs to the repair of drill injuries in the tibia diaphysis of Adiponectin-creERT; tdTomato; Col1(2.3)-GFP mice treated with tamoxifen at 8–12 weeks of age and injured at 16 weeks of age. Adiponectin-creERT recombined in 71 ± 16% of osteoblasts at the injury site 10 days after the drill injury (Figures 3E and 3H).

When we performed a larger drill injury (1.6 mm diameter), we observed similar results: most osteoblasts derived from cells that expressed Adiponectin-cre, Adiponectin-creERT, or LepRcre, and very few osteoblasts derived from cells that expressed Gli1CreERT2 (Figures S6G–S6L). Osteoblasts do not express LepR or Adiponectin (Zhou et al., 2014; Tikhonova et al., 2019; Zhou et al., 2017), and only around 20% of osteoblasts are Tomato+ in the diaphysis of young-adult Adiponectin-cre, LepRcre, or Adiponectin-creERT mice (Figures 1A–1C). While pre-existing osteoblasts may make a small contribution to the repair of bone injuries (Matthews et al., 2021), the approximately 20% of osteoblasts that would have been Tomato+ prior to the injury could not account for the 50%–80% of osteoblasts that were Tomato+ after the repair of the injury. The data, therefore, suggest that uncommitted LepR+/Adiponectin+ bone marrow SSCs are primarily responsible for the repair of drill injuries.

Bicortical fractures are healed primarily by periosteal SSCs

We next performed bicortical fractures in the tibia diaphysis of 10- to 14-week-old Gli1CreERT2; tdTomato; Col1(2.3)-GFP mice, LepRcre; tdTomato; Col1(2.3)-GFP mice, and Adiponectin-cre; tdTomato; Col1(2.3)-GFP mice. These fractures healed over a period of 5 weeks (Figure 4A) through endochondral ossification in which a cartilage callus formed at the fracture site followed by a boney hard callus and, finally, cortical bone and bone marrow stromal cell regeneration (Figures SSA and SSF–SSF) (Colnot, 2009). Both periosteal SSCs and bone marrow SSCs in the diaphysis began to proliferate after the fracture, with approximately 40% incorporating a 48-h pulse of EdU starting 24 h after
We also analyzed the contributions of these cell populations to Col1(2.3)-GFP osteoblasts in the hard callus 14 days after the fracture. In the woven bone of the hard callus, less than 2% of osteoblasts were Tomato+ in Adiponectin-cre; tdTomato mice (Figures 5A and 5D), 15 ± 4.9% were Tomato+ in Leprcre; tdTomato; Col1(2.3)-GFP mice (Figures 5B and 5D), and 43 ± 12% were Tomato+ in Gli1CreERT2; tdTomato; Col1(2.3)-GFP mice (Figures 5C and 5D). Hard callus osteoblasts thus arose primarily from periostial SSCs.

We also observed the formation of new trabecular bone within the bone marrow at the fracture site, which presumably helped to stabilize the fracture. In this trabecular bone, more than 65% of the osteoblasts were Tomato+ in Adiponectin-cre; tdTomato; Col1(2.3)-GFP mice and Leprcre; tdTomato; Col1(2.3)-GFP mice (Figures 5A, 5B, and 5E). Only 8.1 ± 12% of osteoblasts in trabecular bone were Tomato+ in Gli1CreERT2; tdTomato; Col1(2.3)-GFP mice (Figures 5C and 5E). This trabecular bone was thus primarily formed by bone marrow SSCs. Both bone marrow and periostial SSCs thus contributed to the stabilization of the fracture and bone regeneration at early time points after the injury.
At 35 days after the fracture, when it was largely healed, only 1.9 ± 1.5% of the osteocytes in the new cortical bone at the fracture site were Tomato⁺ in Adiponecin-cre; tdTomato mice (Figures 5F and S6D), and 21 ± 15% were Tomato⁺ in Leprcre; tdTomato mice (Figures 5F and S6E). In contrast, 54 ± 13% of new cortical osteocytes at the fracture site were Tomato⁺ in Gli1creERT2; tdTomato mice (Figures 5F and S6F). By day 35, the trabecular bone that had formed within the bone marrow at the fracture site had largely been resorbed. Thus, the new cortical bone that persisted at the healed fracture site was derived mainly from periosteal SSCs.

To test the functional importance of Gli1creERT2; periosteal SSCs for bicortical fracture repair, we generated Gli1creERT2; iDTA mice, in which Gli1creERT2-expressing cells were ablated by expression of the intracellular A subunit of diphtheria toxin (Voehringer et al., 2008). Three days after the last tamoxifen treatment, periosteal CFU-F and Sca1⁺CD51⁺ cells were depleted as compared to littermate controls (Figures 6A and 6B). Following bicortical fracture, these mice had delayed fracture healing, with significantly reduced total callus and callus bone volumes at 14 days (Figures 6C, 6D, and 6F). These data indicate that Gli1⁺ periosteal SSCs contribute functionally to bicortical fracture repair.

We next tested if osteogenic differentiation by periosteal Gli1creERT2⁺ cells contributes to bicortical fracture healing. Wnt/β-catenin signaling promotes osteogenesis during fracture healing (Matsushita et al., 2020; Chen et al., 2007), though to our knowledge it has not yet been tested whether β-catenin is necessary in periosteal SSCs. To test this, we analyzed fracture healing in Gli1creERT2; Ctnnb1flox/flox mice. Thirty-five days after bicortical fracture, the regenerating bone within the fracture callus of Gli1creERT2; Ctnnb1flox/flox mice appeared discontinuous, suggesting incomplete healing (Figure 6G). We quantified bone volume as a fraction of total volume (BV/TV) in the fracture callus and found it was significantly reduced in Gli1creERT2; Ctnnb1flox/flox mice as compared to Ctnnb1flox/flox controls (Figures 6E and 6G). These data further support the functional importance of osteogenesis by Gli1⁺ periosteal cells for fracture repair.

**Periosteal SSCs regenerate LepR⁺ bone marrow stromal cells during fracture repair**

During the healing of bicortical fractures, bone marrow also regenerated at the fracture site at later time points after the resorption of the callus and the trabecular bone that transiently stabilized the fracture at the early stages of repair (Figures 7A and 7B). We thus analyzed the contributions of Adiponecin-creERT-expressing bone marrow cells and Gli1creERT2-expressing periosteal cells to the regeneration of bone marrow stromal cells at the fracture site. Tamoxifen was administered 2–4 weeks before the fracture. In normal adult tibia diaphysis bone marrow, nearly half of LepR⁺ bone marrow stromal cells were Tomato⁺ in Adiponecin-creERT; tdTomato mice (Figures 7C and 1C), but only rare bone marrow stromal cells were Tomato⁺ in Gli1creERT2; tdTomato mice (Figures 7D and 2). Conversely, in bone marrow that regenerated at the fracture site 35 days after the fracture, we observed widespread labeling of bone marrow stromal cells by Gli1creERT2 (Figure 7D) but not by Adiponecin-creERT (Figure 7C). This suggests that bone marrow stromal cells regenerate largely from periosteal SSCs.
The stromal cells that arose from Glit1creERT2-expressing periosteal cells in regenerated bone marrow were perivascular, with an appearance and localization similar to LepR+ stromal cells in normal bone marrow (Figures 7E and 7F). To test whether these cells expressed LepR, we stained sections through regenerated bone marrow in Glit1creERT2; tdTomato mice with anti-LepR antibody. Many Tomato+ perivascular stromal cells stained positively for LepR at 5 weeks (Figure 7E) and 12 weeks (Figure S7E) after fracture. We also stained sections with anti-endomucin antibody. Many Tomato+ perivascular stromal cells stained positively with anti-LepR antibody at 5 to 12 weeks after fracture (Figures S7F). These Tomato+ cells represented 8%–20% of all bone marrow LepR+ cells in the diaphysis of these tibias at 5 to 12 weeks after fracture (Figure 7G). Glit1+ periosteal cells thus regenerate LepR+ bone marrow stromal cells after fracture repair.

We next tested whether the LepR+ bone marrow stromal cells that regenerated from Glit1+ periosteal cells acquire the properties of hematopoietic stem/progenitor cell (HSPC) niche cells. We isolated Tomato+ stromal cells from the tibial diaphysis bone marrow of Glit1creERT2; tdTomato mice 7 weeks after bicornical fracture. SCF and Cxcl12 are niche factors that are strongly expressed by LepR+ bone marrow stromal cells and required for the maintenance of HSPCs (Ding et al., 2012; Ding and Morrison, 2013). We found that Tomato+ stromal cells from the bone marrow of Glit1creERT2; tdTomato tibias lost the expression of Glit1 as compared to periosteal cells (Figure 7J), but began expressing Scf and Cxcl12, genes that exhibit little or no expression in periosteal cells (Figures 7H and 7I). Indeed, 95 ± 0.7% of Tomato+ LepR+ stromal cells from the bone marrow of Glit1creERT2; tdTomato mice were Scf-GFP+ at 5 weeks after the fracture (Figures S7H, 7K, and 7L). Glit1+ periosteal cells thus gave rise to bone marrow stromal cells during bicornical fracture repair that had properties similar to HSPC niche cells, losing the...
expression of Gli1 and acquiring a perivascular localization and expression of Lepr, Scf, and Cxcl12.

**DISCUSSION**

In this study, we confirmed that LepR marks SSCs in adult bone marrow (Figure 1A) and that these cells give rise to most of the osteoblasts and osteocytes that contribute to the maintenance of the adult skeleton under steady-state conditions (Figures 1A and 2M). We also found that LepR bone marrow SSCs are the cells primarily responsible for the repair of drill injuries (Figure 3). However, Gli1+ periosteal SSCs are primarily responsible for the repair of bicortical fractures (Figures 4 and 5). We previously reported that LepR+ bone marrow SSCs produce most of the osteoblasts responsible for fracture repair in adult mice (Zhou et al., 2014). However, in the current study we found that in addition to the SSCs within the bone marrow (Figure 1A), Leprcre also labels a minority of SSCs in the periosteum (Figures 2F–2H). Thus, the contributions of LepR+ cells to fracture repair reflect the activities of both LepR+ bone marrow SSCs and a subpopulation of LepR+ periosteal SSCs. Our results are consistent with recent reports that Leprcre recombines in a subset of SSCs in the periosteum (Ortinau et al., 2019; Mo et al., 2021). In the current study, we confirmed that LepR+ cells within the bone marrow give rise to transient trabecular bone within the bone marrow at the fracture site. LepR+ bone marrow SSCs and Gli1+ periosteal SSCs thus both contribute to the stabilization of bicortical fractures in the first few weeks after injury by forming trabecular bone and cartilage, respectively, at the fracture site.
While Lepr<sup>cre</sup> recombined in bone marrow SSCs and a subset of periosteal SSCs, Adiponectin-<i>cre</i> and Adiponectin-<i>creERT</i> recombined exclusively in bone marrow SSCs. In a prior study, treatment of Adiponectin-<i>creERT</i>; <i>tdTomato</i> mice with low dose tamoxifen recombined in 5% of LepR<sup>+</sup> bone marrow cells that were fated to form adipocytes (Zhou et al., 2017). In the current study, we treated Adiponectin-<i>creERT</i>; <i>tdTomato</i> mice with a much higher dose of tamoxifen, recombining in approximately 40% of LepR<sup>+</sup> bone marrow cells, including in most bone marrow CFU-F (Figure 1C). Consistent with this, nearly all Adiponectin-expressing stromal cells in the bone marrow were LepR<sup>+</sup>, and most LepR<sup>+</sup> stromal cells were Adiponectin<sup>+</sup> (Figures 1D–1F). While Adiponectin is often considered an adipocyte marker, Adiponectin<sup>+</sup>LepR<sup>+</sup> cells do not express other markers of mature adipocytes and do not have large lipid vacuoles, adipocyte size, or morphology. Adiponectin<sup>+</sup> bone marrow stromal cells have been described as marrow adipogenic lineage precursors (MALPs) based on single-cell RNA sequencing (Zhong et al., 2020), but fate mapping shows that these cells are not committed to the adipocyte lineage. Only a subset of LepR<sup>+</sup> stromal cells is fated to form adipocytes (Tikhonova et al., 2019; Baryawno et al., 2020), and even this subset retains the ability to form bone in response to injury (Matsushita et al., 2020). Therefore, most LepR<sup>+</sup>Adiponectin<sup>+</sup> bone marrow stromal cells are not adipocytes or adipocyte-committed progenitors. Bone marrow SSCs are thus reminiscent of other stem cells, such as hematopoietic stem cells (Morrison et al., 1995), that sometimes express markers that are also expressed by their differentiated progeny. Within the periosteum, Sca1<sup>+</sup>CDS1<sup>+</sup> cells were enriched for CFU-F activity (Figure 2B), consistent with prior reports
SSCs in the diaphysis of adult long bones. Within the periosteal compartment, Gi1creERT2 recombined in approximately 40% of CFU-F and Sca1+CD51+ cells (Figures 2J and 2K). This may reflect incomplete recombination among Gi1-expressing periosteal cells (Figure S2C). It is also possible that there is a Gi1-negative subset of periosteal SSCs, potentially with properties that differ from the Gi1+ SSCs.

The LepR+Adiponectin+ bone marrow SSCs and the Gi1+ periosteal SSCs that we studied overlap with previously characterized SSC populations. SSCs have been isolated by flow cytometry from enzymatically dissociated fetal/neonatal and adult bones/bone marrow based on the expression of CD51 and other markers (Chan et al., 2015). The CD51+ SSCs are present in multiple regions of fetal and/or adult bones, including in the peristemeum, bone marrow, and growth plate, but it is not clear if the fates of CD51+ SSCs differ among different regions of bone (Ambrosi et al., 2021a, 2021b).

The mechanisms that ensure different SSC populations repair different kinds of bone injuries remain unclear. Gi1+ periosteal cells and LepR+Adiponectin+ bone marrow stromal cells both begin dividing after drill injuries and bictical fractures (Figures 3C and 4C). Therefore, both SSC populations sense both types of injuries even though the Gi1+ periosteal cells make little contribution to the repair of drill injuries (Figures 3G–3K). Our data show that β-catenin promotes the osteogenic response of Gi1+ periosteal cells to bictical fractures (Figures 6E and 6G). This is consistent with the observation that β-catenin deletion in cells at bictical fracture sites as a result of viral transduction with Cre recombinase impaired fracture healing (Chen et al., 2007). Another recent study demonstrated that deletion of β-catenin from a subset of bone marrow LepR+ cells impaired the repair of drill hole injuries (Matushita et al., 2020). Wnt pathway activation thus appears to promote the osteogenic response of both periosteal and bone marrow SSCs to bone injuries.

In addition to damaging bone, bictical fractures damage the bone marrow at the fracture site. To our knowledge, the process by which bone marrow stroma regenerates at fracture sites has not been studied. Surprisingly, we found that Gi1+ periosteal cells gave rise to LepR+ bone marrow stromal cells in regenerated bone marrow (Figures 7D–7G and S7E–S7H). These LepR+ bone marrow stromal cells that arose from Gi1+ periosteal cells had properties similar to HSPC niche cells in terms of their perivascular localization, their loss of Gi1 expression, and their acquisition of Sca1 and Cxcl12 expression—genes not expressed by periosteal cells (Figures 7H–7J). This suggests that Gi1+ periosteal SSCs were able to transdifferentiate into LepR+ bone marrow SSCs late in the process of bictical fracture repair.

LIMITATIONS OF THE STUDY

The data reported in this study were collected in the femurs and tibias of young-adult mice. Thus, the markers we describe for bone marrow and periosteal SSCs have been verified only in this context. It is possible that these markers are less effective in some other bones or during aging. It is also possible that the functions of bone marrow versus periosteal SSCs differ in some other bones or during aging.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODELS AND SUBJECT DETAILS
  - Mice
- METHOD DETAILS
  - Drill injuries and fractures
  - Tissue dissociation
  - Flow cytometry
  - Cell proliferation
  - Immunostaining of bone sections
  - Safranin O with fast green staining
  - Hematoxylin and eosin (H&E) staining
  - CFU-F assay
  - Quantitative RT-PCR
  - MicroCT analysis
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Quantification of Tomato+ cells in confocal images
  - Single cell RNA-sequencing analysis
  - Statistical methods

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.stem.2022.10.002.

ACKNOWLEDGMENTS

S.J.M. is a Howard Hughes Medical Institute (HHMI) investigator, the Mary McDermott Cook Chair in Pediatric Genetics, the Kathryn and Gene Bishop Distinguished Chair in Pediatric Research, the director of the Hamon Laboratory for Stem Cells and Cancer, and a Cancer Prevention and Research Institute of Texas Scholar. This work was supported partly by the Josephine Hughes Sterling Foundation. E.C.J. was supported by a postdoctoral fellowship from the Damon Runyon Cancer Research Foundation (2278–16). We thank B. Levi and R. Zondervan for use of their bone fracture apparatus. We thank N. Loof, T. Shih, and C. Cantu of the Moody Foundation Flow Cytometry Facility, as well as the BioHPC high performance computing cloud at UT Southwestern Medical Center. We thank S.J. Conway for Periostin-cre mice and Y. Wan for CathepsinKCre mice. This article is subject to HHMI’s Open Access to Publications policy. HHMI lab heads have previously granted a nonexclusive CC BY 4.0 license to the public and a sublicensable license to HHMI in their research articles. Pursuant to those licenses, the author-accepted manuscript of this article can be made freely available under a CC BY 4.0 license immediately upon publication.

AUTHOR CONTRIBUTIONS

E.C.J. and S.J.M. conceived the project, designed, and interpreted experiments. E.C.J. performed most of the experiments, with technical assistance from T.L.A.M. and J.A.P. Z.Z. analyzed single-cell RNA sequencing data and assisted with statistical analyses. E.C.J. and S.J.M. wrote the manuscript.
Cell Stem Cell

DECLARATION OF INTERESTS
The authors declare no competing interests.

Received: March 15, 2022
Revised: September 15, 2022
Accepted: October 6, 2022
Published: October 21, 2022

REFERENCES

Duchamp de Lageneoste, O., Julien, A., Abou-Khalil, R., Frangi, G., Carvalho, C., Cagnard, N., Cordier, C., Conway, S.J., and Colnot, C. (2018). Periosteum contains skeletal stem cells with high bone regenerative potential controlled by Periostin. Nat. Commun. 9, 773. https://doi.org/10.1038/s41467-018-03124-2.

Debnath, S., Yallowitz, A.R., McCormick, J., Lalani, S., Zhang, T., Xu, R., Li, N., Liu, Y., Yang, Y.S., Eisenman, M., et al. (2018). Discovery of a periosteal cell mediating intramembranous bone formation. Nature 562, 133–139. https://doi.org/10.1038/s41586-018-0554-8.

Ortinau, L.C., Wang, H., Lei, K., Deveza, L., Jeong, Y., Hara, Y., Grafe, I., Rosenfeld, S.B., Lee, D., Lee, B., et al. (2019). Identification of Functionally Distinct Mx1+vs Mx1− Periosteal Skeletal Stem Cells. Cell Stem Cell 25, 784–796.e5. https://doi.org/10.1016/j.stem.2019.11.003.

Matthews, B.G., Novak, S., Sbrana, F.V., Funnell, J.L., Cao, Y., Buckels, E.J., Grcievic, D., and Kalajzic, I. (2021). Heterogeneity of murine periosteum progenitors involved in fracture healing. Elife 10, e58534. https://doi.org/10.7554/elife.58534.

Zhang, X., Naik, A., Xie, C., Reynolds, D., Palmer, J., Lin, A., Awad, H., Guilberg, R., Schwarz, E., and O’Keefe, R. (2005). Periosteal stem cells are essential for bone regeneration and repair. J. Musculoskelet. Neuronal Interact. 5, 360–362.

Braut, V., Moore, R., Kutsch, S., Ishibashi, M., Rowitch, D.H., McMahon, A.P., Sommer, L., Bousadia, O., and Kemler, R. (2001). Inactivation of the beta-catenin gene by Wnt1-Cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development. Development 128, 1253–1264. https://doi.org/10.1242/dev.128.8.1253.

Chan, C., Seo, E., Chen, J., Lo, D., Mcardle, A., Sinha, R., Tevlin, R., Seita, J., Vincent-Tompkins, J., Wearda, T., et al. (2015). Identification and specification of the mouse skeletal stem cell. Cell 160, 285–298. https://doi.org/10.1016/j.cell.2014.12.002.

Zhou, B., Yue, R., Murphy, M., Peyer, J.G., and Morrison, S.J. (2014). Leptin-receptor-expressing mesenchymal stromal cells represent the main source of osteoprogenitors in bone formed by adult bone marrow. Cell Stem Cell 15, 154–168. https://doi.org/10.1016/j.stem.2014.06.008.

Matsushita, Y., Sagawa, T., Hara, H., Kohno, K., and Nagasawa, T. (2010). The essential functions of adipogenic progenitors as the hematopoietic stem and progenitor cell niche. Immunity 33, 387–399. https://doi.org/10.1016/j.immuni.2010.08.017.

Ding, L., Saunders, T.L., Enikolopov, G., and Morrison, S.J. (2012). Endothelial and perivascular cells maintain haematopoietic stem cells. Nature 481, 457–462. https://doi.org/10.1038/nature10783.

Omatsu, Y., Sugiyama, T., Kohara, H., Kondoh, G., Fujii, N., Kohno, K., and Nagasawa, T. (2010). The essential functions of adipogenic progenitors as the hematopoietic stem and progenitor cell niche. Immunity 33, 387–399. https://doi.org/10.1016/j.immuni.2010.08.017.

Ding, L., and Morrison, S.J. (2013). Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. Nature 495, 231–235. https://doi.org/10.1038/nature11885.

Maes, C., Kobayashi, T., Selig, M.K., Torrekeens, S., Roth, S.J., Mackern, S., Carmeliet, G., and Kronenberg, H.M. (2010). Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels. Dev. Cell 19, 329–344. https://doi.org/10.1016/j.devcel.2010.07.010.

Shu, H.-S., Liu, Y.-L., Tang, X.T., Zhang, X.S., Zhou, B., Zou, W., and Zhou, B.O. (2021). Tracing the skeletal progenitor transition during postnatal bone formation. Cell Stem Cell 28, 2122–2136.e3. https://doi.org/10.1016/j.stem.2021.08.010.

Tikhonova, A.N., Dolgalev, I., Hu, H., Sivaraj, K.K., Hoek, E., Cuesta-Dominguez, A., Pinho, S., Akhmetzyanova, I., Gao, J., Witkowski, M., et al. (2019). The bone marrow microenvironment at single-cell resolution. Nature 569, 222–228. https://doi.org/10.1038/s41586-019-1104-8.

Baryawno, N., Przybylski, D., Kowalczyk, M.S., Kfouri, Y., Hote, N., Gustafsson, K., Kokkaliaris, K.D., Mercier, F., Tabaka, M., Hofree, M., Dione, D., Papazian, A., Lee, D., Ashenberg, O., Subramaniam, A., Vaishnav, E.D., Rosenblatt-Rosen, O., Regev, A., and Scadden, D.T. (2019). A Cellular Taxonomy of the Bone Marrow Stroma in Homeostasis and Leukemia. Cell 177, 1915–1932.e16. e1916. https://doi.org/10.1016/j.cell.2019.04.040.

Please cite this article in press as: Jeffery et al., Bone marrow and periosteal skeletal stem/progenitor cells make distinct contributions to bone maintenance and repair, Cell Stem Cell (2022), https://doi.org/10.1016/j.stem.2022.10.002
Baccin, C., Al-Sabah, J., Velten, L., Hebling, P.M., Grunschlager, F., Hernandez-Malmierca, P., Nombela-Arrieta, C., Steinmetz, L.M., Trump, A., and Haas, S. (2020). Combined single-cell and spatial transcriptomics reveal the molecular, cellular and spatial bone marrow niche organization. Nat. Cell Biol. 22, 38–48. https://doi.org/10.1038/s41556-019-0439-6.

Zhou, B.O., Yu, H., Yue, R., Zhao, Z., Rios, J.J., Naveiras, O., and Morrison, S.J. (2017). Bone marrow adipocytes promote the regeneration of stem cells and haematopoiesis by secreting SCF. Nat. Cell Biol. 19, 891–903. https://doi.org/10.1038/ncb3570.

Shi, Y., He, G., Lee, W.C., McKenzie, J.A., Silva, M.J., and Long, F. (2017). Gli1 identifies osteogenic progenitors for bone formation and fracture repair. Nat. Commun. 8, 2043. https://doi.org/10.1038/s41467-017-02171-2.

Zhao, H., Feng, J., Ho, T.V., Grimes, W., Urata, M., and Chai, Y. (2015). The suture provides a niche for mesenchymal stem cells of craniofacial bones. Nat. Cell Biol. 17, 386–396. https://doi.org/10.1038/ncb3139.

Xu, J., Wang, Y., Li, Z., Tian, Y., Li, Z., Lu, A., Hsu, C.Y., Negri, S., Tang, C., Tower, R.J., et al. (2022). PDGFRα reporter activity identifies periosseous progenitor cells critical for bone formation and fracture repair. Bone Res 10, 7. https://doi.org/10.1038/s41414-021-00176-8.

Chang, H., and Knottie Tate, M.L. (2012). Concise review: the periosteum: tapping into a reservoir of clinically useful progenitor cells. Stem Cells Transl Med 1, 480–481. https://doi.org/10.5966/sctm.2011-0056.

Ito, Y., Fitzsimmons, J.S., Sanyal, A., Mello, M.A., Mukherjee, N., and Driscoll, S.W. (2001). Localization of chondrocyte precursors in periosteum. Osteoarthritis Cartilage 9, 215–223. https://doi.org/10.1038/sj.oac.1600376.

Coint, N. (2009). Skeletal cell fate decisions within periosteum and bone marrow during bone regeneration. J. Bone Miner. Res. 24, 274–282. https://doi.org/10.1002/jbmr.801003.

Matthews, B.G., Torregiani, E., Roeder, E., Matic, I., Greveci, D., and Kalapcz, I. (2016). Osteogenic potential of alpha smooth muscle actin expressing muscle resident progenitor cells. Bone 84, 69–77. https://doi.org/10.1016/j.bone.2015.12.010.

Kawamura, A., Matsushita, T., Chan, Y.Y., and Murakami, S. (2009). Mice expressing GFP and CreER in osteochondro progenitor cells in the periosteum. Biochem. Biophys. Res. Commun. 386, 477–482. https://doi.org/10.1016/j.bbrc.2009.06.059.

Murao, H., Yamamoto, M., Matsuoka, S., and Akiyama, H. (2013). Periosteal cells are a major source of soft callus in bone fracture. J Bone Miner Metab 31, 390–398. https://doi.org/10.1007/s00774-013-0429-x.

Mo, C., Guo, J., Qin, J., Zhang, X., Sun, Y., Wei, H., Cao, D., Zhang, Y., Zhao, C., Xiong, Y., et al. (2022). Single-cell transcriptomics of LepR-positive skeletal cells reveals heterogeneous stress-dependent stem and progenitor pools. EMBO J. 47, e108415. https://doi.org/10.15252/embj.2021108415.

Logan, M., Martin, J.F., Nagy, A., Lobe, C., Olson, E.N., and Tabin, C.J. (2002). Expression of Cre Recombinase in the developing mouse limb bud driven by a Prx1 enhancer. Genesis 33, 77–80. https://doi.org/10.1002/gene.10092.

Greenbaum, A., Hsu, Y.M.S., Day, R.B., Schuettgelz, L.G., Christopher, M.J., Borgerding, J., Nagasawa, T., and Link, D.C. (2013). CXCL12 in early fracture callus is essential for bone fracture healing. J. Clin. Investig. 123, 477–482. https://doi.org/10.1172/JCI61502.

Jaiyeola, C., Zhao, Z., Luby-Phelps, K., and Morrison, S.J. (2015). Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal. Nature 526, 126–130. https://doi.org/10.1038/nature15250.

Horiuchi, K., Amizuka, N., Takeshita, S., Takamatsu, H., Katsuura, M., Ozawa, H., Toyama, Y., Bonefeld, L.F., and Kudo, A. (1999). Identification and characterization of a novel protein, peristin, with restricted expression to perisarticular bones. Nat. Cell Biol. 1, 77–80. https://doi.org/10.1038/nd10092.

Green, A.C., Tijin, G., Lee, S.C., Chak, A.M., Straszkowski, L., Kwang, D., Baker, E.K., Quach, J.M., Kimura, T., Wu, J.Y., and Purton, L.E. (2021). The characterization of distinct populations of murine skeletal cells that have different roles in B lymphopoiesis. Blood 138, 304–317. https://doi.org/10.1182/blood.2020005865.

Acar, M., Kocherlakota, K.S., Murphy, M.M., Peyer, J.G., Oguro, H., Inra, C.N., Jayeola, C., Zhao, Z., Luby-Phelps, K., and Morrison, S.J. (2015). Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal. Nature 526, 126–130. https://doi.org/10.1038/nature15250.

Wendling, O., Bornert, J.M., Champon, P., and Metzger, D. (2009). Efficient temporally-controlled targeted mutagenesis in smooth muscle cells of the adult mouse. Genesis 47, 14–18. https://doi.org/10.1002/dvg.20448.

Voehringer, D., Liang, H.E., and Locksley, R.M. (2008). Homeostasis and effector function of lymphopoiesis-induced ‘memory-like’ T cells in constitutively T cell-depleted mice. J. Immunol. 180, 4742–4753. https://doi.org/10.4049/jimmunol.180.7.4742.

Chen, Y., Whetstone, H.C., Lin, A.C., Nadenos, P., Wei, O., Poon, R., and Alman, B.A. (2007). Beta-catenin signaling plays a disparate role in different phases of fracture repair: implications for therapy to improve bone healing. PLoS Med. 4, e249. https://doi.org/10.1371/journal.pmed.0040249.

Kusumbe, A.P., Ramasamy, S.K., and Adams, R.H. (2014). Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. Nature 507, 323–328. https://doi.org/10.1038/nature13145.

Morrison, S.J., Hemmati, H.D., Wandycz, A.M., and Weissman, I.L. (1995). The purification and characterization of fetal liver hematopoietic stem cells. Proc. Natl. Acad. Sci. USA. 92, 10302–10306. https://doi.org/10.1073/pnas.92.22.10302.

Ambrosi, T.H., Sinha, R., Steininger, H.M., Hoover, M.Y., Murphy, M.P., Koepke, L.S., Wang, Y., Lu, W.J., Morri, M., Neff, N.F., et al. (2021). Distinct skeletal stem cell types orchestrate long bone skeletogenesis. Elife 10, e66083. https://doi.org/10.7554/elife.66083.

Ahn, S., and Joyner, A.L. (2004). Dynamic changes in the response of cells to positive hedgehog signaling during mouse limb patterning. Cell 118, 505–516. https://doi.org/10.1016/j.cell.2004.07.023.
Ambrosi, T.H., Marecic, O., McArdle, A., Sinha, R., Gulati, G.S., Tong, X., Wang, Y., Steininger, H.M., Hoover, M.Y., Koepke, L.S., et al. (2021). Aged skeletal stem cells generate an inflammatory degenerative niche. Nature 597, 256–262. https://doi.org/10.1038/s41586-021-03795-7.

DeFalco, J., Tomishima, M., Liu, H., Zhao, C., Cai, X., Marth, J.D., Enquist, L., and Friedman, J.M. (2001). Virus-assisted mapping of neural inputs to a feeding center in the hypothalamus. Science 291, 2608–2613. https://doi.org/10.1126/science.1056602.

Madisen, L., Zwingman, T.A., Sunkin, S.M., Oh, S.W., Zariwala, H.A., Gu, H., Ng, L.L., Palmiter, R.D., Hawrylycz, M.J., Jones, A.R., et al. (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat. Neurosci. 13, 133–140. https://doi.org/10.1038/nn.2487.

Nakamura, T., Imai, Y., Matsumoto, T., Sato, S., Takeuchi, K., Igarashi, K., Harada, Y., Azuma, Y., Krust, A., Yamamoto, Y., et al. (2007). Estrogen prevents bone loss via estrogen receptor alpha and induction of Fas ligand in osteoclasts. Cell 130, 811–823. https://doi.org/10.1016/j.cell.2007.07.025.

Jeffery, E., Church, C.D., Holtrup, B., Colman, L., and Rodeheffer, M.S. (2015). Rapid depot-specific activation of adipocyte precursor cells at the onset of obesity. Nat Cell Biol 17, 376–385. https://doi.org/10.1038/nclb3122.

Joseph, N.M., Mesher, J.T., Buchstaller, J., Snider, P., McKeever, P.E., Lim, M., Conway, S.J., Parada, L.F., Zhu, Y., and Morrison, S.J. (2008). The loss of Nf1 transiently promotes self-renewal but not tumorigenesis by neural crest stem cells. Cancer Cell 13, 129–140. https://doi.org/10.1016/j.ccr.2008.01.003.

Kuhn, R., Schwenk, F., Aguet, M., and Rajewsky, K. (1995). Inducible gene targeting in mice. Science 269, 1427–1429. https://doi.org/10.1126/science.7660125.

Balordi, F., and Fishell, G. (2007). Mosaic removal of hedgehog signaling in the adult SVZ reveals that the residual wild-type stem cells have a limited capacity for self-renewal. J. Neurosci. 27, 14248–14259. https://doi.org/10.1523/JNEUROSCI.4531-07.2007.

Zondervan, R.L., Vorce, M., Servadio, N., and Hankenson, K.D. (2018). Fracture Apparatus Design and Protocol Optimization for Closed-stabilized Fractures in Rodents. J Vis Exp. https://doi.org/10.3791/58186.

Morrison, S.J., Csete, M., Groves, A.K., Melega, W., Wold, B., and Anderson, D.J. (2000). Culture in reduced levels of oxygen promotes clonogenic sympathetic differentiation by isolated neural crest stem cells. J. Neurosci. 20, 7370–7376. https://doi.org/10.1523/jneurosci.20-19-07370.2000.

Stuart, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck, W.M., 3rd, Hao, Y., Stoeckius, M., Smibert, P., and Satija, R. (2019). Comprehensive Integration of Single-Cell Data. Cell 177, 1888–1902.e21. https://doi.org/10.1016/j.cell.2019.05.031.
### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies** | | |
| z-mouse Ter119 APC | Tonbo Biosciences | clone TER-119; RRID: AB_2621609 |
| z-mouse CD45 APC | Tonbo Biosciences | clone 30-F11; RRID: AB_2621573 |
| z-mouse Sca1 Alexa Fluor 700 | eBioscience | clone D7; RRID: AB_657837 |
| z-mouse CD51-biotin | BioLegend | clone RMV-7; RRID: AB_313073 |
| Goat z-mouse LepR antibody, Biotin | R&D Systems | BAF497; RRID: AB_2296953 |
| z-mouse VE-cadherin (CD144) efluor660 | eBioscience | clone BV13; RRID: AB_11219483 |
| Streptavidin APC-efluor780 | eBioscience | Cat. 47-4317-82; RRID: AB_10366688 |
| Streptavidin Brilliant Violet 421 | BioLegend | Cat.405226 |
| Goat anti-tdTomato | LSBio | Cat. LS-C340696; RRID: AB_2819022 |
| Chicken anti-GFP | Aves Labs | Cat. GFP-1020; RRID: AB_10000240 |
| rabbit z-dsRed/tdTomato antibody | Takara | Cat. 632496; RRID: AB_10013483 |
| Rabbit anti-aggrecan | EMD Millipore | Cat. AB1031; RRID: AB_90460 |
| Goat anti-sclerostin | R&D Systems | Cat. AF1589; RRID: AB_2195344 |
| Goat anti-endomucin | R&D Systems | Cat. AF4666; RRID: AB_2100035 |
| Rabbit anti-laminin 1&2 | Abcam | Cat. AB7463; RRID: AB_305933 |
| Goat anti-periostin | R&D Systems | Cat. AF2955; RRID: AB_664123 |
| Goat anti-Gii1 | R&D Systems | Cat. AF3455; RRID: AB_2247710 |
| Rabbit anti-CD51 | Abcam | Cat. ab179475; RRID: AB_2716738 |
| Goat anti-Sca1 | R&D Systems | Cat. AF1226; RRID: AB_354679 |
| Goat IgG | R&D Systems | Cat. AB-108-C; RRID: AB_354267 |
| Rabbit IgG | R&D Systems | Cat. AB-105-C; RRID: AB_354266 |
| Chicken IgY | R&D Systems | Cat. AB-101-C; RRID: AB_354263 |
| F(ab')₂ Fragment Donkey Anti-Rabbit IgG (H + L) Alexa Fluor 647 | Jackson ImmunoResearch | Cat. 711-606-152; RRID: AB_2340625 |
| F(ab')₂ Fragment Donkey Anti-Chicken IgY (H + L) Alexa Fluor 488 | Jackson ImmunoResearch | Cat. 703-546-155; RRID: AB_2340376 |
| F(ab')₂ Fragment Donkey Anti-Goat IgG (H + L) Alexa Fluor 647 | Jackson ImmunoResearch | Cat. 705-606-147; RRID: AB_2340438 |
| Donkey Anti-Goat IgG Polyclonal Antibody CF-555 | Biotium | Cat. 20039; RRID: AB_10556967 |
| Donkey Anti-Rabbit IgG Polyclonal Antibody CF-555 | Biotium | Cat. 20038; RRID: AB_10558011 |

| **Chemicals, Peptides, and Recombinant Proteins** | | |
| HI bovine serum (FBS) | Thermo Fisher Scientific | Cat. 26170043 |
| Hank’s Balanced Salt Solution (HBSS) | Thermo Fisher Scientific | Cat. MT21022CV |
| Collagenase I | Sigma-Aldrich | Cat. 10104159001 |
| Collagenase D | Worthington Biochemical | Cat. LS004197 |
| Dispase II | Sigma-Aldrich | Cat. 11088866001 |
| 5-ethyl-2'-deoxyuridine | Thermo Fisher Scientific | Cat. A10044 |
| NP-40 | Sigma- Aldrich | Cat. I8896 |
| Donkey serum | Jackson ImmunoResearch | Cat. 017-000-121 |
| DMSO | Sigma-Aldrich | Cat. D4540 |
| ROCK inhibitor Y-27632 | Tocris | Cat. 1254 |
| DMEM (high glucose) | Gibco | Cat. 11965118 |
| Penicillin/Streptomycin | Fisher Scientific | Cat. SV30010 |

(Continued on next page)
Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Fetal Bovine Serum  | Sigma-Aldrich | Cat F0926 |
| Toluidine blue O    | Sigma-Aldrich | Cat. T3260 |
| 4% Paraformaldehyde | Fisher Scientific | Cat. AAJ19943K2 |
| Hematoxylin QS Counterstain | Vector Laboratories | Cat. H-3404-100 |
| Bluing buffer       | DAKO   | Cat. CD702 |
| Prolong Gold anti-fade reagent | ThermoFisher | Cat. P36930 |
| Weigert’s iron hematoxylin | Sigma-Aldrich | Cat. 1.15973 |
| Fast Green FCF      | Sigma-Aldrich | Cat. C.1 42053 |
| Acetic acid         | Sigma-Aldrich | Cat. TMS-009 |
| Safranin O          | Sigma-Aldrich | Cat. T5648 |
| Eosin Y             | Sigma-Aldrich | Cat. C8267 |
| 10% Formalin        | Sigma-Aldrich | Cat. 23-245-685 |
| Tamoxifen           | Sigma-Aldrich | Cat. 1.15973 |
| Corn oil            | Sigma-Aldrich | Cat. 1.15973 |
| Buprenorphine SR    | ZooPharm LLC | Prescription only |
| Isoflurane          | Covetrus | SKU 029405 |

Critical Commercial Assays

| Critical Commercial Assays | SOURCE | IDENTIFIER |
|---------------------------|--------|------------|
| Click-it Edu Alexa Fluor 647 Flow cytometry kit | Thermo Fisher Scientific | Cat. C10424 |
| Click-it Edu Alexa Fluor 647 Imaging kit | Thermo Fisher Scientific | Cat. C10340 |
| Qiagen RNeasy Micro Kit | Qiagen | Cat. 74004 |
| iScript cDNA Synthesis Kit | Bio-Rad Laboratories | Cat. 170-8891 |
| iTaq Universal SYBR Green Supermix | Bio-Rad Laboratories | Cat. 172-5124 |

Deposited Data

| Deposited Data | SOURCE | IDENTIFIER |
|----------------|--------|------------|
| Single cell RNAseq dataset | Mo et al., 2021 | Geo accession# GSE138689 |

Experimental Models: Organisms/Strains

| LepR-Cre (B6.129(Cg)-Leptrtm1(cre)Rcn/J, RRID:IMSR_JAX:008,320) | DeFalco et al., 2001 | JAX: 008320 |
| Rs26-tdTomato (B6.Cg-Gt(Rosa)26Sortm9(CAG-tdTomato)Hze/J, RRID:IMSR_JAX:007,909) | Jackson Laboratory | JAX: 007909 |
| Adiponectin-cre (B6.FVB-Tg(Adipoq-cre)1Evd/J, RRID:IMSR_JAX:028,020) | Eguchi et al., 2011 | JAX: 028020 |
| Adiponectin-creERT (B6.129-Tg(Adipoq-cre/Esr1)1Evd/J, RRID:IMSR_JAX:024,671) | Jeffery et al., 2015 | JAX: 024671 |
| Col2a1+2.3-EGFP (Tg(Col1a1+2.3-EGFP)1Rowe) | Kalajzic et al., 2002 | MGI: 4353048 |
| Gli1creERT2 (Gli1tm1(creERT2)Alj/J, RRID:IMSR_JAX:007,913) | Ahn and Joyner, 2004 | JAX: 007913 |
| nSMA-creERT2 (Tg(Acta2-cre/ERT2)#Pcn) | Wendling et al., 2009 | MGI: 5050721 |
| CathepsinKcre (Ctsktm1(cre)Ska) | Nakamura et al., 2007 | MGI: 3764465 |
| Periostin-cre (Tg(Postn-cre)1Sjc) | Joseph et al., 2008 | MGI: 3775923 |
| Mx1-cre (B6.Cg-Tg(Mx1-cre)1Cgn/J, RRID:IMSR_JAX:003,556) | Kuhn at al., 1995 | JAX: 003556 |
| Gremlin1-creERT (B6.Cg-Tg(Gremlin1-cre/ERT2)3Tcw/J, RRID:IMSR_JAX:027,039) | Worthley et al., 2015 | JAX: 027039 |
| Osterix-creERT2 (Tg(Sp7-Tg(Grem1-cre/ERT2)3Tcw/J, RRID:IMSR_JAX:027,039) | Maes et al., 2010 | MGI: 4829803 |
| Nestin-creER | Balordi and Fishell, 2007 | N/A |
| Ctnnb1flax (Ctnnb1tm2(Kem)) | Brault et al., 2001 | JAX: 004152 |
| Rosa-iDTA (B6.129P2-Gt(Rosa)26Sortm1(cre)Dvlk/J, RRID:IMSR_JAX:009,669) | Voehringer et al., 2008 | JAX: 009669 |
| SCF-GFP (STOCK Kltm1.1Sl/J, RRID:IMSR_JAX:017,860) | Ding et al., 2012 | JAX: 017860 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sean Morrison (sean.morrison@utsouthwestern.edu).

Materials availability
This study did not generate new unique reagents.

Data and code availability
Microscopy and flow cytometry data reported in this paper will be shared by the lead contact upon request. This paper analyzes existing, publicly available data. The accession numbers for the datasets are listed in the key resources table. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Mice
All mouse experiments complied with all relevant ethical regulations and were performed according to a protocol approved by the Institutional Animal Care and Use Committee at UT Southwestern Medical Center (protocol 2017–101896). All mice were maintained on a C57BL/Ka background, including Lepcre (DeFalco et al., 2001), Rosa26-CAG-loxp-stop-loxp-tdTomato (tdTomato) (Madisen et al., 2010), Col1a1*2.3-GFP (Kalajzic et al., 2002), Gli1creERT2 (Ahn and Joyner, 2004) (Jax #007913), Adiponectin-cre (Eguchi et al., 2011) (Jax #028020), Adiponectin-creERT (Jeffery et al., 2015) (Jax #024671), αSMA-creERT2 (Wendling et al., 2009), CathepsinKcre (Nakamura et al., 2007), Periostin-cre (Joseph et al., 2009), Mx1-cre (Kuhn et al., 1995), Gremlin1-creERT2 (Jax #027039), Nestin-creER (Balordi and Fishell, 2007), Osterix-creERT2 (Maes et al., 2010), Ctnnb1floxF (Brault et al., 2001) (Jax #004152), SCF-GFP (Jax #017860), and Rosa-DTA (Jax #009669). Mice were genotyped using primers listed in Table S1. To induce Cre recombination, unless otherwise indicated, CreER expressing mice were gavaged with 6 mg of tamoxifen dissolved in corn oil every other day for 7 days, except Adiponectin-creERT mice, which were instead given tamoxifen-containing chow (ENVIGO TD.130860) for 4 weeks, followed by 4 weeks on regular chow, before analysis. Both male and female mice were used in this study, except for experiments involving microCT-based quantification of bone healing in which case only female mice were used. All mice were housed in specific pathogen-free animal care facilities under a 12h:12h light/dark cycle with a temperature of 65–75°F and a humidity of 40–60%.

METHOD DETAILS

Drill injuries and fractures
For drill injuries, mice were anesthetized with isoflurane (Covetrus), a small incision was made in the skin over the central region of the tibia diaphysis and a micro dental drill with 0.8 mm or 1.6 mm diameter was used to make a hole through one side of the tibia cortex, exposing the bone marrow. The skin was closed with 4/0 sutures (Covetrus) and 1.2 mg/kg of Buprenorphine SR (ZooPharm) was injected for analgesia. For bicortical fractures, a 27 gauge needle was inserted into the intramedullary canal of the tibia through the knee joint after anesthesia, and the tibia was fractured mid-diaphysis by 3-point bending using a Zondervan apparatus (Zondervan et al., 2018). The location of the pin was verified by X-ray radiography on an AMI HTX Optical Imaging System (Spectral Instruments Imaging). Buprenorphine SR was injected immediately after the surgery, while the mice were still anesthetized.

Tissue dissociation
To isolate bone marrow cells, bones were thoroughly cleaned to remove periosteal cells. The metaphyses of the femur and/or tibia were separated from the diaphysis with dissection scissors, and the diaphysis was flushed with dissociation buffer using a 25-gauge syringe and 27-gauge needle.
needle. The diaphysis bone was then crushed and combined with the flushed marrow for dissociation. The metaphyses were separately crushed in dissociation buffer using a mortar and pestle, and the contents were transferred to a tube for digestion. Bone marrow dissociation buffer contained type I collagenase (3 mg/mL, Worthington Biochemical), dispase (4 mg/mL, Sigma-Aldrich) and DNase I (1 U/mL, Roche) in Hank’s balanced salt solution (HBSS) with Ca²⁺ and Mg²⁺ (Corning). Samples were dissociated at 37°C for 30 min as described previously (Yue et al., 2016). Dissociated cells were then transferred into HBSS (without Ca²⁺ and Mg²⁺) plus 2% serum, centrifuged, then resuspended in HBSS (without Ca²⁺ and Mg²⁺) plus 2% serum and filtered through 40μm mesh to generate a single cell suspension.

To isolate periosteal cells, muscle was carefully trimmed from femurs and tibias. Bones were placed in HBSS (without Ca²⁺ and Mg²⁺) plus 2% serum on ice for 30 min to loosen the periosteal layer from the bone surface. Periosteum was then peeled from the long bones, placed on a nylon mesh filter, and washed with cold HBSS (with Ca²⁺ and Mg²⁺) to wash away contaminating bone marrow cells. Chunks of periosteum were then placed in periosteum dissociation buffer containing collagenase D (1 mg/mL, Roche), dispase (2 mg/mL, Sigma-Aldrich) and DNase I (1 U/mL, Roche) in HBSS with Ca²⁺ and Mg²⁺ (Corning). Samples were dissociated at 37°C for 45 min. The dissociated cells were then transferred into HBSS (without Ca²⁺ and Mg²⁺) plus 2% serum, washed by centrifugation and resuspension, and filtered through a 40μm mesh to generate a single cell suspension.

To isolate cells from fractured bones, the fractured region was separated from the rest of the bone with dissection scissors, then crushed in dissociation buffer using a mortar and pestle and transferred to a tube for dissociation. The fractured bone dissociation buffer contained collagenase D (1 mg/mL, Roche), type I collagenase (3 mg/mL, Worthington Biochemical), dispase (4 mg/mL, Sigma-Aldrich) and DNase I (1 U/mL, Roche) in HBSS with Ca²⁺ and Mg²⁺ (Corning). Samples were dissociated at 37°C for 45 min. Dissociated cells were then transferred into HBSS (without Ca²⁺ and Mg²⁺) plus 2% serum, washed by centrifugation and resuspension, and filtered through a 40μm mesh to generate a single cell suspension.

Flow cytometry

Samples were stained with combinations of the following antibodies for flow cytometry: Goat-anti-LepR-biotin (AF497, R&D Systems), anti-CD45 (30F-11, Tonbo Biosciences), anti-CD144 (clone BV13, eBiosciences), anti-TER119 (Tonbo Biosciences), anti-Sca1(D7, eBiosciences), anti-CD51-biotin (RMV-7, BioLegend), Streptavidin BV421 (BioLegend), and/or Streptavidin APCeFluor 780 (eBiosciences). All staining was performed for 1.5 h on ice. Dead cells were identified and gated out of sorts by including 4’,6-diamidino-2-phenylindole (DAPI) (1 μg/mL) in the buffer in which cells were resuspended for flow cytometry or by staining cells with Ghost Dye Red 780 (1:100, Tonbo Biosciences) before resuspending cells for flow cytometric analysis. Samples were analyzed or sorted using FACSaria flow cytometers and FACSDiva 8.0 (BD) or FlowJo v10.6.1 (Tree Star) software.

Cell proliferation

To analyze cell proliferation after drill injury or fracture, mice were injected intraperitoneally with 1 mg of 5-ethynyl-2'-deoxyuridine (EdU) (A10044, Thermo Fisher Scientific) dissolved in PBS at 24 and 48 h after injury. Mice were euthanized at 72 h after injury for flow cytometric analysis or 96 h for bone sectioning. The injured and contralateral control tibias were dissociated as described above for flow cytometric analysis, or fixed in 4% paraformaldehyde for sectioning. Flow cytometry samples were processed for EdU staining using the Click-it EdU Alexa Fluor 647 Flow Cytometry Kit (C10424, Invitrogen) according to the manufacturer’s instructions. Bones used for sectioning were processed as described below and stained for EdU with the Click-it EdU Alexa Fluor 647 Imaging Kit (C10340, Invitrogen) according to the manufacturer’s instructions prior to staining with other antibodies.

Immunostaining of bone sections

Freshly dissected mouse femurs or tibias were fixed in 4% paraformaldehyde for 6 to 16 h at 4°C, then washed several times in PBS. Bones were decalcified in PBS (PBS) with 10% EDTA and 30% sucrose for 2–4 weeks with constant agitation. Bones were sectioned in 10μm slices using the CryoJane tape-transfer system (Leica). Sections were permeabized for 10 min with 0.05% NP40 and 2.5% DMSO in HBSS, then washed in PBS and blocked in PBS with 5% normal donkey serum (Jackson Immunoresearch) for 1 h. Slides were then stained overnight with combinations of the following antibodies: chicken anti-GFP (1–200, Aves Labs, GFP-1020), rabbit anti-tomato (1–200, Takara, 632,496), goat anti-dsRed (1–200, LifeSpan Biosciences, LS-C340696), rabbit anti-Aggrecan (1–35, EMD Millipore, AB1031), goat anti-Sclerostin (1–35, R&D Systems, AF1589), goat anti-endomucin (1–200, R&D Systems, AF4666), or goat anti-LepR-biotin (1–50, AF497, R&D Systems), rabbit anti-laminin (1–200, AB7463, Abcam), goat anti-periostin (1–100, AF2955, R&D Systems), goat anti-Gli1 (1–10, AF3455,R&D Systems) rabbit anti-CD51 (1–10, ab179475, Abcam), goat anti-Sca1 (1–10, AF1226, R&D Systems R&D Systems). Secondary antibodies included donkey anti-chicken Alexa Fluor 488 (1–250, Jackson Immunoresearch), donkey anti-goat Alexa Fluor 647 (1–250, Jackson Immunoresearch), donkey anti-rabbit Alexa Fluor 647 (1–250, Jackson Immunoresearch), donkey anti-goat CF-555 (1–500, Biotium), and donkey anti-rabbit CF-555 (1–500, Biotium). Sections were counterstained with DAPI to stain nuclei before mounting. Slides were mounted with Prolong Gold anti-fade reagent (Invitrogen). Images were acquired with a Zeiss LSM880 confocal microscope using the tiling function, and tiled images were then stitched together using Zen software (Zeiss) to create a composite image of the tissue. Stitched images are indicated in the figure legends. Confocal images were processed and analyzed using Fiji (ImageJ) and Photoshop (Adobe Systems).
Safranin O with fast green staining

Frozen sections were brough to room temperature. Sections were then stained with Weigert’s iron hematoxylin (#1.15973, Sigma-Aldrich) for 10 min. Sections were washed in distilled water 3 times for 5 min each. Sections were then stained with fast green FCF (#C1.42053, Sigma-Aldrich) for 5 min, then rinsed quickly with 1% acetic acid (#A38-212, Fisher) diluted in distilled water. Finally, sections were stained with 0.1% Safranin O (#TMS-009, Sigma-Aldrich) solution for 5 min and washed briefly in distilled water 3 times.

Hematoxylin and eosin (H&E) staining

Frozen sections were treated with Hematoxylin QS Counterstain (#H-3404-100, Vector Laboratories), followed by three washes in distilled water. Sections were then treated briefly with bluing buffer (#CD702, DAKO), followed by three washes in distilled water. Finally, sections were treated briefly with Eosin Y solution (#HT110116, Sigma) followed by three washes in distilled water.

CFU-F assay

Freshly dissociated bone marrow cells that were unfractionated or double sorted for combinations of cell surface markers were plated at clonal density in 6-well plates such that individual CFU-F could form spatially distinct colonies that could be counted. Unfractionated bone marrow was plated at 100,000 cells per well, and unfractionated periosteum was plated at 1000 cells per well in 6-well plates. These cell numbers led to the formation of 30–50 spatially distinct fibroblast colonies per well. The cells were cultured in DMEM with high glucose (Gibco) plus 20% fetal bovine serum (Sigma), 10μM ROCK inhibitor Y-27632 (Tocris, 1254), and 1% penicillin/streptomycin (Invitrogen) at 37°C in gas-tight chambers (Billups-Rothenberg) flushed with 1% O2 and 6% CO2 (balance Nitrogen) to maintain physiological oxygen levels that promoted survival and proliferation (Morrison et al., 2000). The medium was changed the next day to eliminate contaminating hematopoietic cells and dead cells. To count the colonies, the cultures were stained with 0.1% Toluidine blue in 10% formalin eight days after plating and the percentage of colonies that was Tomato+ was assessed by direct fluorescence (without antibody staining) using an Olympus IX83 inverted microscope.

Quantitative RT-PCR

Cells were sorted directly into 75μL of Buffer RLT lysis buffer (Qiagen) containing 1% β-mercaptoethanol and stored at –80°C. RNA was extracted using the Qiagen RNeasy Micro Kit (74,004, Qiagen) according to the manufacturer’s instructions. The eluted RNA was then used to make cDNA using iScript cDNA Synthesis Kit (#170–8891, Bio-Rad Laboratories) according to the manufacturer’s instructions. cDNA was then used to perform qPCR with iTaq Universal SYBR Green Supermix (#172–5124, Bio-Rad Laboratories) and results were analyzed using BioRad CFX Maestro Software.

MicroCT analysis

Tibias from mice at 0 to 35 days after drill injury or 7 to 35 days after fracture were fixed in 4% paraformaldehyde overnight at 4°C, washed with PBS, and scanned using a Scanco Medical μCT 35. Tibias were scanned at an isotropic voxel size of 3.5μm, with peak tube voltage of 55 kV and current of 0.145 mA (μCT 35; Scanco). A three-dimensional Gaussian filter (σ = 0.8) with a limited, finite filter support of one was used to suppress noise. A threshold of 342–1000 was used to segment mineralized bone from air and soft tissues. To determine callus volume, Scanco Medical software was used to draw contours around the outside of the callus in the x-y plane on every 10th level on the z axis, and the morph function was used to interpolate contours for the intervening levels. Callus total volume and bone volume were then determined using the same number of z-slices for each bone in the experiment. Analysis of bone volume as a fraction of total volume (BV/TV) was performed using the entire callus volume for each bone.

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification of Tomato+ cells in confocal images

To quantitate the percentage of cells within sections that were Tomato+, relevant representative regions from confocal images were chosen from multiple areas of the bone or the bone callus and at least 150 cells (osteoblasts and chondrocytes) or 80 cells (osteocytes) were counted. First, we counted the total number of osteoblasts/chondrocytes/osteocytes in the field of view in the absence of Tomato fluorescence, then the Tomato channel was turned on and the percentage that were Tomato+ was determined. This procedure was repeated for each mouse.

Single cell RNA-sequencing analysis

Single-cell RNA sequencing data from the Gene Expression Omnibus (GEO) study GSE138689 were used for the analysis (Mo et al., 2021). Samples’ barcodes, features, and matrix files were downloaded from the study’s GEO entry and processed using R 4.0.2 with the Seurat 3.2 package (Stuart et al., 2019). Low-quality cells with >10% of their transcripts from mitochondrial genes, <100 Unique Molecular Identifier (UMI) counts, or <100 genes per cell were excluded from analysis. Filtered samples were then normalized and integrated using Seurat’s variance stabilizing transformations for single-cell UMI data (SCTransform). Clustering was performed, and samples and genes were visualized using Seurat as well.
Statistical methods

In each type of experiment, multiple mice were tested, generally in multiple independent experiments performed on different days. Mice were allocated to experiments randomly and samples were processed in an arbitrary order, but formal randomization and blinding techniques were not used. Prior to analyzing the statistical significance of differences among treatments, we tested whether the data were normally distributed and whether variance was similar among treatments. To test for normal distribution, we performed the Shapiro-Wilk test when $3 \leq n < 20$ or the D’Agostino Omnibus test when $n \geq 20$. To test if variability significantly differed among treatments, we performed $F$-tests (for experiments with two treatments) or Levene’s median tests (for more than two treatments). When the data significantly deviated from normality or variability significantly differed among treatments, we log2-transformed the data and tested again for normality and variability. If the transformed data no longer significantly deviated from normality and equal variability, we performed parametric tests on the transformed data. If the transformed data still significantly deviated from normality or equal variability, we performed non-parametric tests on the non-transformed data.

All the statistical tests we used were two-sided, where applicable. To assess the statistical significance of a difference between two treatments, we used t-tests (when mice were not littermates and a parametric test was appropriate), or Mann-Whitney tests (when mice were not littermates and a non-parametric test was appropriate). To assess the statistical significance of differences between more than two treatments, we used repeated measures one-way or two-way ANOVAs (when mice were littermates and/or cells were from same mice, and a parametric test was appropriate) followed by Tukey’s, Dunnet’s, or Sidak’s multiple comparisons adjustment. All statistical analyses were performed with Graphpad Prism 8.3 or R 3.5.1 with the fBasics package. All data represent mean ± SD. Samples sizes were not pre-determined based on statistical power calculations.
Supplemental Information

Bone marrow and periosteal skeletal stem/progenitor cells make distinct contributions to bone maintenance and repair

Elise C. Jeffery, Terry L.A. Mann, Jade A. Pool, Zhiyu Zhao, and Sean J. Morrison
Supplementary Figure 1. *Nestin-creER, Gremlin1-creERT2, Osterix-creERT2* and *Gli1creERT2* exhibit little recombination in SSCs within the bone marrow of adult mice, particularly in the diaphysis of long bones. Related to Figure 1. (A–D) Each row shows the percentage of bone marrow stromal cells (negative for CD45, Ter119, and VE-cadherin), LepR⁺ bone marrow stromal cells, Col1(2.3)-GFP⁺ endosteal osteoblasts, and CFU-F that were Tomato⁺ in *Nestin-creER; tdTomato; Col1(2.3)-GFP* (A), *Gremlin1-creERT2; tdTomato; Col1(2.3)-GFP* (B), *Osterix-creER; tdTomato; Col1(2.3)-GFP* (C) or *Gli1creERT2; tdTomato; Col1(2.3)-GFP* (D) mice. The number of mice analyzed per time point is shown in each panel. Tamoxifen was administered at 8-9 weeks of age. *Osterix-creERT2* mice received 2 doses of 4mg of tamoxifen on consecutive days. All other mice received 4 doses of 6 mg of tamoxifen every other day. All data represent mean ± standard deviation.
Supplementary Figure 2. Adiponectin$^+$ cells are perivascular in the bone marrow and Ctsk$^{cre}$, Mx1-cre, Periostin-cre, and $\alpha$SMA-creERT2 are not effective markers for periosteal SSCs in adult mice. Related to Figure 2. (A) Representative confocal images from the diaphysis of long bones from Adiponectin-cre; tdTomato (top panel) or Adiponectin-creERT; tdTomato (bottom panel) adult mice stained with an antibody against laminin to identify the basement membranes around blood vessels. White arrows indicate perisinusoidal cells and yellow arrows indicate periarteriolar cells. Data are representative of images taken from 3 mice per genotype. (B) Representative confocal images of the tibial periosteum stained with antibodies against Sca1 and CD51. White arrows indicate Sca1$^+$CD51$^+$ cells in the cambial layer and yellow arrows indicate Sca1$^+$CD51$^+$ cells in the fibrous layer. Data are representative of images taken from 2 mice. The lower magnification images in the top row of panel B were acquired as tiled images and stitched together. (C-F) Each row shows the percentage of stromal cells (negative for CD45, Ter119, and VE-cadherin) in the periosteum or in the bone marrow diaphysis or metaphysis that were Tomato$^+$, the percentage of LepR$^+$ bone marrow stromal cells that were Tomato$^+$, and the percentage of CFU-F in the periosteum or bone marrow diaphysis or metaphysis that were Tomato$^+$ in Ctsk$^{cre}$; tdTomato (C), Mx1-cre; tdTomato (D), Periostin-cre; tdTomato (E), or $\alpha$SMA-creERT2; tdTomato (F) mice. In each case, the cells were obtained from the femurs and tibias of 8-10 week old mice. (G) Representative confocal images of sections cut from long bones of 8 week old Ctsk$^{cre}$; tdTomato mice stained with anti-LepR antibody. Arrows indicate Tomato$^+$LepR$^+$ cells. Data are representative of images taken from 3 mice. Each dot represents a different mouse, analyzed in 1 to 3 experiments per genetic background. $\alpha$SMA-creERT2 mice received 2 doses of 4mg tamoxifen on consecutive days. All data represent mean ± standard deviation.
**Supplementary Figure 3**

**A** Pre-injury tamoxifen

| Tam | Tam | Tam | Tam | Day 0 | Day 10 |
|-----|-----|-----|-----|-------|--------|
| day -13 | day -11 | day -9 | day -7 | Drill | Analyze |

**B** Post-injury tamoxifen

| Tam | Tam | Tam |
|-----|-----|-----|
| Day 0 | Day 1 | Day 10 |
| Drill | Analyze |

**C** Gli1creERT2 ;tdTomato
Post drill injury day 10

Tomato  DAPI

**D** αSMA-creERT2 ; tdTomato
Post drill injury day 10

Tomato  DAPI

**E** Day 10 post drill injury

αSMA-creERT2 ; tdTomato

- Bone marrow
- 100% of osteoblasts

**F** Gli1creERT2 ; tdTomato
Post fracture day 7

Tomato  DAPI

**G** αSMA-creERT2 ; tdTomato
Post fracture day 7

Tomato  DAPI

**H** Day 7 post fracture

αSMA-creERT2 ; tdTomato

- Callus
- Bone marrow
- 100% of chondrocytes

**I** Day 7 post fracture

αSMA-creERT2 ; tdTomato

- Callus
- Bone marrow
- 100% of osteoblasts
Supplementary Figure 3. \(\alpha\text{SMA-creERT2}\) labels cells in both the bone marrow and periosteum after bone injuries. Related to Figure 2. (A-B) Experimental timelines for pre-injury (A) or post-injury (B) tamoxifen treatment. Pre-injury tamoxifen was administered to \(Gli1^{\text{creERT2}}\) mice, while post-injury tamoxifen was administered to \(\alpha\text{SMA-creERT2}\) mice in certain experiments. (C-D) Representative confocal images of tibias from \(Gli1^{\text{creERT2}}; tdTomato\) (C) or \(\alpha\text{SMA-creERT2}; tdTomato\) (D) mice 10 days after drill injury. Tamoxifen was administered to \(\alpha\text{SMA-creERT2}; tdTomato\) mice on days 0 and 1 after injury. Data are representative of 3-4 mice per genotype. (E) The percentage of \(\text{Col1}(2.3)-\text{GFP}^+\) osteoblasts in the drill injury region that were Tomato\(^+\) at 10 days after injury. (F-G) Representative confocal images of tibias from \(Gli1^{\text{creERT2}}; tdTomato\) (F) or \(\alpha\text{SMA-creERT2}; tdTomato\) (G) mice 7 days after bicortical fracture. Tamoxifen was administered to \(\alpha\text{SMA-creERT2}; tdTomato\) mice on days 0 and 1 after fracture. (H) The percentage of Aggrecan\(^+\) chondrocytes that were Tomato\(^+\) in the fracture callus at 7 days after fracture. (I) The percentage of \(\text{Col1}(2.3)-\text{GFP}^+\) osteoblasts in the bone marrow or fracture callus that were Tomato\(^+\) at 10 days after the fracture. Tomato\(^+\) cells were present in both the bone marrow and the periosteum near the sites of bone injuries in \(\alpha\text{SMA-creERT2}; tdTomato\) mice, while Tomato\(^+\) cells were restricted to the periosteum and bone callus in \(Gli1^{\text{creERT2}}; tdTomato\) mice. All images in this figure were acquired as tiled images and stitched together. Each dot represents a different mouse, analyzed in 1 to 2 experiments per genetic background. All data represent mean ± standard deviation.
Supplementary Figure 4

A. UMAP 1 and UMAP 2

B. Expression level of SCF across clusters

C. Expression level of SCF across clusters

D. Expression level of Cxcl12 across clusters

E. Expression level of Acta2 across different conditions

F. Immunofluorescence images of bone, periosteum, and muscle

G. Immunofluorescence images of bone, periosteum, and muscle

H. Expression of Gli1 relative to β-actin

I. Immunofluorescence images of Gli1 and DAPI

J. Immunofluorescence images of goat anti-Gli1 and dTomato

Legend:
- SS: Steady state
- Frac: Fracture
- Irr: Irradiated

- 0: Lepr, Wisp2
- 1: SCF, Cxcl12, BMP4
- 2: Grem1, Wisp2
- 3: SCF, Adipoq, Cxcl12
- 4: Col2a1, Postn, Ctsk
- 5: Cxcl12, Adipoq
- 6: Alpl, Witt1, Clec11a
- 7: Acta2
- 8: Fatp4
- 9: Sp7, Col1a1, Ctsk
- 10: Ccl2, Sca1
- 11: Il7
- 12: Plin1, FABP4, Pparg

- Bone marrow LepR+ cells
- Gli1creERT2; tdTomato
- Sca1+CD51+
- periosteal cells
- Gli1creERT2; tdTomato- Sca1+CD51+
- periosteal cells

- Goat IgG
- Goat anti-Gli1
Supplementary Figure 4. The gene that encodes Smooth Muscle Actin, *Acta2*, is induced in periosteal cells and LepR<sup>+</sup> bone marrow stromal cells after bicortical fractures and cells expressing *Lepr<sup>cre</sup>* and *Gli1<sup>creERT2</sup>* are located in both the cambial and fibrous layers of the periosteum. Related to Figure 2. (A) Reanalysis of single cell RNA sequencing data from ref<sup>39</sup> showing cell clusters and marker genes expressed by Tomato<sup>+</sup> cells isolated by flow cytometry from enzymatically dissociated bone/bone marrow cells obtained from *Lepr<sup>cre</sup>*; *tdTomato* mice at steady-state, 7 days after fracture, or 7 days after irradiation. (B-D) Expression levels of genes expressed by HSC niche cells in the bone marrow in cell clusters from (A). (E) Expression level of *Acta2* in each cell cluster from control, fractured, or irradiated mice. (F-G) Confocal images of tibia sections from *Gli1<sup>creERT2</sup>*; *tdTomato* (F) and *Lepr<sup>cre</sup>*; *tdTomato* (G) mice stained with an antibody against periostin to identify the cambial and fibrous layers of the periosteum. White arrows indicate Tomato<sup>+</sup> cells in the cambial layer and yellow arrows indicate Tomato<sup>+</sup> cells in the fibrous layer. Data are representative of images taken from 3 mice per genotype. (H) qRT-PCR analysis of *Gli1* expression in sorted Sca1<sup>+</sup>CD51<sup>+</sup>Tomato<sup>+</sup> and Sca1<sup>+</sup>CD51<sup>+</sup>Tomato-negative cells from the periosteum of *Gli1<sup>creERT2</sup>*; *tdTomato* mice as well as sorted LepR<sup>+</sup> bone marrow cells identified based on antibody staining. Cells were isolated 3 days after the completion of tamoxifen treatment. Each dot represents a different mouse. (I-J) Confocal images of tibia sections from *Lepr<sup>cre</sup>*; *tdTomato* mice stained with an antibody against Gli1 or IgG isotype control. Data are representative of images taken from 2 mice. White arrows indicate Tomato<sup>+</sup>Gli1<sup>+</sup> cells. Note that the muscle adjacent to the periosteum is autofluorescent in the green channel. All data represent mean ± standard deviation. Statistical significance in (H) was assessed using multiple or paired t-tests followed by Holm-Sidak’s adjustment for multiple comparisons.
Supplementary Figure 5

A. Bicortical fracture day 7
   Safranin O Fast Green

B. 0.8mm drill injury day 2
   Safranin O Fast Green

C. 0.8mm drill injury day 10
   Safranin O Fast Green

D. 1.6mm drill injury day 10
   Safranin O Fast Green

E. Hematoxylin and Eosin
   Post drill injury day 10

F. Post bicortical fracture day 7

G. Post bicortical fracture day 14

H. Post bicortical fracture day 35

Legend:
- Bone marrow
- Drill injury site
- Cartilage callus
- Pin insertion site
- Fracture site
- Regenerated bone marrow
- Hard callus
Supplementary Figure 5. Bicortical fractures heal via endochondral ossification and drill injuries heal via intramembranous ossification. Related to Figure 3. (A-D) Staining of tibia cryosections with Safranin O and fast green to label cartilage (red). (E-H) Staining of tibia cryosections with hematoxylin and eosin to provide orientation for the time points analyzed by immunofluorescence.
Supplementary Figure 6

**Post drill injury day 35**

A. Adipoq-cre

B. Leprcre

C. Gli1creERT2

**Post bicortical fracture day 35**

D. Adipoq-cre

E. Leprcre

F. Gli1creERT2

---

**Large (1.6mm) drill hole injury**

G. Day 0 Day 10

H. Day 10 post injury

---

I. Post large drill hole injury day 10

J. Adipoq-creERT

K. Leprcre

L. Gli1creERT2

---

H. % of osteoblasts

- Adipoq-cre
- Adipoq-creERT
- Leprcre
- Gli1creERT2
Supplementary Figure 6. Osteocytes in the regenerated bone cortex after drill hole injuries or bicortical fractures are labelled by Adiponectin-cre or Gli1creERT2, respectively, and bone marrow SSCs form most of the osteoblasts that contribute to the repair of large drill hole injuries. Related to Figure 3. (A-C) Representative confocal images of Tomato+ osteocytes (yellow arrows) in regenerated cortical bone 35 days after drill injuries in 17-19 week old Adiponectin-cre; tdTomato (A), Leprcre; tdTomato (B), and Gli1creERT2; tdTomato (C) mice. The images are representative of 4 (Adiponectin-cre) or 3 (Leprcre and Gli1creERT2) mice. The percentages of osteocytes that were Tomato+ in these mice is shown in Figure 3K. The lower magnification images in the left-most panels in A-C were acquired as tiled images and stitched together. (D-F) Representative confocal images of Tomato+ osteocytes (yellow arrows) in regenerated cortical bone in tibias 35 days after biocortical fractures in 17-19 week old Adiponectin-cre; tdTomato (D), Leprcre; tdTomato (E), and Gli1creERT2; tdTomato (F) mice. The images are representative of 4 (Adiponectin-cre and Leprcre) or 8 (Gli1creERT2) mice. The percentages of osteocytes that were Tomato+ in these mice is shown in Figure 5F. (G) microCT images of tibias from mice that received a drill injury. (H) The percentage of Col1(2.3)-GFP+ osteoblasts in the drill injury region that were Tomato+ at 10 days after injury. (I-L) Representative confocal images of tibias from 12-14 week old Adiponectin-cre; tdTomato (I), Adiponectin-creERT; tdTomato (J), Leprcre; tdTomato (K), and Gli1creERT2; tdTomato (L) mice 10 days after the drill injury. Data are representative of images from 3 (Adiponectin-cre) or 4 mice (Adiponectin-creERT, Leprcre and Gli1creERT2) per genotype. The lower magnification images in the left-most panels in I-L were acquired as tiled images and stitched together. Each dot represents a different mouse. Statistical significance in (H) was assessed using a one-way ANOVA followed by Tukey’s multiple comparisons correction.
Supplementary Figure 7

A. Post fracture day 4

B. Unfractured

C. Fractured

D. Unfractured

E. Fractured

F. Gli1creERT2 ;tdTomato

G. Bone marrow, Post fracture week 7

H. Gli1creERT2 ;tdTomato; SCF-GFP

I. Bone marrow, Post fracture week 5
Supplementary Figure 7. Tomato+ cells from the periosteum of Gli1\textsuperscript{creERT2}; tdTomato mice and the bone marrow of Adiponectin\textsuperscript{cre}; tdTomato mice were quiescent under steady-state conditions but went into cycle after bicortical fractures and Gli1\textsuperscript{creERT2}; tdTomato+ cells give rise to LepR+ bone marrow stromal cells that express HSC niche factors in regenerated bone marrow. Related to Figures 4 and 7 (A-D) Confocal images of sections from unfractured (A and C) or fractured (B and D) tibias of Gli1\textsuperscript{creERT2}; tdTomato or Adiponectin-cre; tdTomato mice that were treated with EdU at 24 and 48 hours after bicortical fracture, then analyzed at 96 hours after the fracture. Arrows indicate EdU+Tomato+ cells. Data are representative of images from 3 mice per group. The lower magnification images in the left-most panels in (A-D) were acquired as tiled images and stitched together. (E) Confocal images of regenerated bones from Gli1\textsuperscript{creERT2}; tdTomato mice stained with an antibody against LepR 12 weeks after bicortical fracture. Arrows indicate Tomato+LepR+ cells. (F-G) Representative flow cytometric analysis of bone marrow isolated from tibias of Gli1\textsuperscript{creERT2}; tdTomato mice at week 7 (F) or 12 (G) after fracture, and stained with an antibody against LepR. Data are representative of 3 mice per group. (H) Representative flow cytometric analysis of bone marrow from Gli1\textsuperscript{creERT2}; tdTomato; SCF-GFP mice at 5 weeks after bicortical fracture showing that bone marrow stromal cells derived from Gli1+ periosteal cells express LepR and Scf. Data are representative of 2 mice. All data represent mean ± standard deviation.
| Gene               | Primer sequence (5'-3') |
|--------------------|-------------------------|
| Leprcre P1         | CATTGTATGGGATCTGATCTGG  |
| Leprcre P2         | CCTGAACATGTCCATCAGGTTCTT |
| Leprcre P3         | GTTGGATGAGCTTTTGGAACGTA |
| Leprcre P4         | AGAACACATTATACATGACAGGCT|
| Cre P1             | GCGGTCTGGCGAGTAAACACTATC|
| Cre P2             | GTGAAACAGCACTTTCGTGCTACTT|
| CreER P1           | GTGCCTTGGCTAGAGATCCTTG  |
| CreER P2           | AGAGACTTCAAGGTTGCTGGA   |
| Tomato P1          | AAGGGAGCTGCAGTGGAGTA    |
| Tomato P2          | CCGAAATCTGTGGAGAAGTC    |
| Tomato P3          | GGCATTAAGCAAGCTATCC     |
| Tomato P4          | CTGTTTCTGTACGGCATGG     |
| Col-GFP P1         | AATCAAGAAACTGCTCTCAGTG1 |
| Col-GFP P2         | TGATATAGACGTTGTGGCTGTTG|
| Ctnnb1 flox P1     | AAGGTAGAGTGATGAAAGTGT   |
| Ctnnb1 flox P2     | CACCATGTCCTCTGTCATTTC   |
| SCF-GFP P1         | CCCGCAGCTCTGATATTTGC    |
| SCF-GFP P2         | CGGACACGCTGAACTTGTGG    |
| SCF-GFP P3         | AAGCACTCATGATTTGTGG     |
| Rosa-DTA P1        | CGACCTGCAGGTCTCTCG      |
| Rosa-DTA P2        | CTCGAGTTTGTCCCAATTATGTCAC|
| Rosa-DTA P3        | CCAAGTCGCTGACTGTTATAC   |
| Rosa-DTA P4        | GAGCGGGAGAAATGGATATG    |

**Supplementary Table 1.** Primers used for genotyping of mice.  
Related to STAR Methods section "Mice".
### Table S2

| Gene   | Primer sequence (5'-3') |
|--------|-------------------------|
| Gli1 P1 | CTCGACCTGCAAACCGTAATC   |
| Gli1 P2 | TCCTAAAGAAGGGCTCATGGTA  |
| bActin P1 | CACTGTGAGTCCGCTGTC   |
| bActin P2 | TCATCCATGGCGAAGCTGTC    |

**Supplementary Table 2. Primers used for quantitative RT-PCR analyses.**

Related to STAR Methods section "Quantitative RT-PCR".