A Wnt-mediated transformation of the bone marrow stromal cell identity orchestrates skeletal regeneration

Yuki Matsushita
*University of Michigan-Ann Arbor*

Mizuki Nagata
*University of Michigan-Ann Arbor*

Kenneth M. Kozloff
*University of Michigan-Ann Arbor*

Joshua D. Welch
*University of Michigan-Ann Arbor*

Koji Mizuhashi
*University of Michigan-Ann Arbor*

See next page for additional authors

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A Wnt-mediated transformation of the bone marrow stromal cell identity orchestrates skeletal regeneration

Yuki Matsushita1, Mizuki Nagata1, Kenneth M. Kozloff2, Joshua D. Welch3, Koji Mizuhashi1, Nicha Tokavanich1, Shawn A. Hallett1, Daniel C. Link4, Takashi Nagasawa5, Wanida Ono1 & Noriaki Ono1*

Bone marrow stromal cells (BMSCs) are versatile mesenchymal cell populations underpinning the major functions of the skeleton, a majority of which adjoin sinusoidal blood vessels and express C-X-C motif chemokine ligand 12 (CXCL12). However, how these cells are activated during regeneration and facilitate osteogenesis remains largely unknown. Cell-lineage analysis using Cxcl12-creER mice reveals that quiescent Cxcl12-creER+ perisinusoidal BMSCs differentiate into cortical bone osteoblasts solely during regeneration. A combined single cell RNA-seq analysis demonstrate that these cells convert their identity into a skeletal stem cell-like state in response to injury, associated with upregulation of osteoblast-signature genes and activation of canonical Wnt signaling components along the single-cell trajectory. β-catenin deficiency in these cells indeed causes insufficiency in cortical bone regeneration. Therefore, quiescent Cxcl12-creER+ BMSCs transform into osteoblast precursor cells in a manner mediated by canonical Wnt signaling, highlighting a unique mechanism by which dormant stromal cells are enlisted for skeletal regeneration.

1University of Michigan School of Dentistry, Ann Arbor, MI 48109, USA. 2Department of Orthopaedic Surgery, University of Michigan, Ann Arbor, MI 48109, USA. 3Department of Computational Medicine and Bioinformatics, Department of Computer Science and Engineering, University of Michigan, Ann Arbor, MI 48109, USA. 4Washington University School of Medicine, Division of Oncology, St. Louis, MO 63110, USA. 5Osaka University School of Medicine, Laboratory of Stem Cell Biology and Developmental Immunology, Graduate School of Frontier Biosciences, Suita, Osaka 565-0871, Japan.
*email: noriono@umich.edu
Bone is a highly regenerative organ that maintains its structural integrity and functionality throughout life. Bone marrow stromal cells (BMSCs) are mesenchymal cell populations with versatile functions that provide the support for hematopoietic cells and the source of osteoblasts, underpinning the major functions of the skeleton. A majority of BMSCs assume perivascular locations surrounding sinusoids with a reticular morphology and universally express cytokines such as C-C motif chemokine 12 (CXCL12, also known as stromal cell-derived factor 1, SDF1) and stem cell factor (SCF, also known as KIT ligand). Leptin receptor (LepR) is expressed by a wide array of BMSCs that largely overlap with CXCL12-abundant reticular (CAR) cells. These LepR+CXCL12- BMSCs populate the osteoblast compartment in adult mice and are self-renewing populations particularly those termed as skeletal stem cells (SSCs) defined by in vitro assays. However, how specific subsets of CXCL12- BMSCs are activated in response to injury within their native environment and facilitate skeletal regeneration remains largely unknown. In this study, we sought to identify the mechanism underlying the remarkable osteogenic capacity of CXCL12- BMSCs in vivo under regenerative conditions. To this end, we generated a Cxcl12-creER line and performed in vivo lineage-tracing experiments and functional analyses. Our data reveal that quiescent Cxcl12-creER- BMSCs transform into precursor cells characterized by a SSC-like state in a manner mediated by canonical Wnt signaling during injury responses, and functionally contribute to skeletal regeneration.

**Results**

**Cxcl12-creER marks a quiescent subset of CXCL12+LepR+ BMSCs.** To reveal in vivo cell fates of CXCL12+ BMSCs, we generated a tamoxifen-inducible Cxcl12-creER bacterial artificial chromosome (BAC) transgenic line (L289, Fig. 1a). First, we characterized this Cxcl12-creER line based on a short-chase protocol. Analysis of Cxcl12-GFP+/–; Cxcl12-creER; R26RtdTomato mice at postnatal day (P) 28 after a tamoxifen pulse at P21 revealed that Cxcl12-creER-marked tdTomato+ (hereafter, Cxcl12-CE-tdTomato) cells overlapped completely with Cxcl12-GFP+stromal cells throughout the diaphyseal marrow space (Fig. 1b). These Cxcl12-GFP+CXCL12+tdTomato+ stromal cells assumed a characteristic morphology as reticular cells adjoining endomucin (Emcn)+ sinusoidal endothelial cells (Fig. 1c, Supplementary Fig. 1a) and CD31+ arteriolar endothelial cells (Fig. 1d). In fact, virtually all Cxcl12-GFP+CXCL12+tdTomato+ cells attached Emcn+ sinusoids either through dendrites or by cell bodies (Fig. 1e, 95.7 ± 2.1% of Cxcl12-GFP+CXCL12+tdTomato+ cells, compared to 84.6 ± 6.1% of Cxcl12-GFP+tdTomato− cells, n = 5). Flow cytometry analysis revealed that Cxcl12-CE-tdTomato+ cells represented an extremely small fraction of whole bone marrow cells (0.014 ± 0.002%). Cxcl12-creER faithfully marked a subset of Cxcl12-GFP+ cells upon tamoxifen injection; 79.7 ± 3.0% of CD45/Ter119/CD31negCXcl12-GFP+ cells were tdTomato+, whereas 97.6 ± 1.1% of CD45/Ter119/CD31negCXcl12-CFSE+tdTomato+ cells were Cxcl12-GFP+ (n = 5, Fig. 1f, Supplementary Fig. 8a, b). Cxcl12-CFSE-tdTomato+ cells ubiquitously expressed leptin receptor (LepR) (Fig. 1g, Supplementary Fig. 8a, b). Notably, Cxcl12-CFSE-tdTomato+ cells were preferentially localized in the central marrow space (Fig. 1h). In fact, Cxcl12-CFSE-tdTomato+ cells accounted for a majority of Cxcl12-GFP+ and LepR+ stromal cells in the central marrow space (tdTomato+: 70.9 ± 15.5% and 70.3 ± 15.2% of total Cxcl12-GFP+ and LepR+ cells, respectively). By contrast, Cxcl12-CFSE-tdTomato+ cells accounted for only a small portion of Cxcl12-GFP+ and LepR+ stromal cells in the metaphyseal marrow space (tdTomato+: 3.6 ± 2.4% and 2.6 ± 1.7% of total Cxcl12-GFP+ and LepR+ cells, respectively) or in the endosteal space (tdTomato+: 16.1 ± 6.2% and 12.6 ± 5.9% of total Cxcl12-GFP+ and LepR+ cells, respectively, Fig. 1i), suggesting that Cxcl12-CFSE-tdTomato+ cells represent a specific subset of LepR+CXCL12+ stromal cells that are distant from the bone surface. Moreover, Cxcl12-creER marked a subset of Cxcl12-GFP+ cells that were characterized by the least mitotic activity and the most abundant expression of CXCL12 but not SCF (Fig. 1j–m, Supplementary Fig. 8a, b). Cxcl12-CFSE-tdTomato+ cells were distinct from mature osteoblasts, as they did not express Cola1(2.3 kb)-GFP (Fig. 1n, o, Supplementary Fig. 8a, b). Importantly, this Cxcl12-creER line had minimal promiscuity in the stromal cell compartment; although tdTomato+ cells were occasionally present in Cxcl12-creER; R26RtdTomato mice in the absence of tamoxifen (6/13 mice, 46%, Supplementary Fig. 1b, c), virtually all of them were in a CD45/Ter119/CD31+ fraction (90.9 ± 3.3%, Supplementary Figs. 1b, 8a). Therefore, Cxcl12-creER can mark a relatively quiescent subset of CXCL12+ peri-sinusoidal BMSCs in the central marrow space upon tamoxifen injection.

**Single-cell characterization of Cxcl12-creER+ BMSCs.** We further defined the identity of Cxcl12-creER+ stromal cells by a single cell RNA-seq analysis. To this end, we interrogated the profile of fluorescently sorted single cells gated on a GFP+ fraction (Supplementary Fig. 8c, d) isolated from Cxcl12-GFP+/–; Cxcl12-creER; R26RtdTomato bone marrow at P28 after a tamoxifen pulse at P21 (Mouse #1: 2749 cells, Mouse #2: 4577 cells, total 7326 cells). Initial analysis revealed that a substantial fraction of cells (Mouse #1: 721/2749 cells, 26.2%, Mouse #2: 747/4577 cells, 16.3%) belonged to the clusters without detectable eGFP expression; these eGFPneg clusters included myeloid cells, lymphocytes, and erythrocytoid cells (Supplementary Fig. 2), highlighting an issue on hematopoietic cell contamination commonly observed in recently published bone marrow stromal datasets. eGFP was exclusively expressed by cells that abundantly expressed Cxcl12 (Supplementary Fig. 2). Cxcl12-GFP+ cells were heterogeneous and clustered into nine groups, including three clusters of stromal (Clusters 0–2), two clusters endothelial (Clusters 4 and 8), one cluster of perioseal (Cluster 3) cells (Fig. 2a, Supplementary Fig. 2). Other small clusters included cells in cell cycle (Cluster 6) and enriched for mitochondrial (Cluster 5) and ribosomal (Cluster 7) genes. The stromal clusters were composed of a reticular cell group expressing pre-adipocyte markers such as Adipoq and Lepr (Cluster 0), and a group expressing pre-osteoblast markers such as Alpl and Posn (Cluster 1) (Fig. 2a, Supplementary Fig. 2). Cells in Cluster 0 were relatively enriched for secreted factors such as Cxcl12, Kitl, Fgf7, and Iil7, whereas those in Cluster 1 were relatively enriched for an osteogenic factor Clec11a (Fig. 2b). Importantly, tdTomato was identified as a cell-type specific marker for Cluster 0 (Fig. 2a, b). Thus, a majority of Cxcl12-CFSE-tdTomato+ cells belongs to a reticular cluster in which cells abundantly express adipocyte-related genes and a variety of cytokines.

We next examined ex vivo properties of Cxcl12-creER+ stromal cells by a colony-forming unit fibroblast (CFU-F) assay (Supplementary Fig. 3a). To achieve this, we plated whole bone marrow cells isolated from Cxcl12-creER; R26RtdTomato mice at P28 (tamoxifen pulse at P21) at a clonal density. Cxcl12-creER marked only a small fraction of CFU-Fs (3.7 ± 0.8%, compared to 99.7 ± 0.6% of total CFU-Fs by ubiquitous Ubc-creER, Fig. 2c, d); this is in sharp contrast with LepR-cre and Ebf3-creER that can mark essentially all CFU-Fs. Therefore, Cxcl12-creER can...
specifically mark a subset of CXCL12+ BMSCs with little colony-forming activities upon tamoxifen injection.

Subsequently, we tested in vitro passageability of individual tdTomato+ clones (Supplementary Fig. 3a). Cxcl12CE-tdTomato+ clones could survive for significantly higher passages than UbcCE-tdTomato+ clones did; while 36.8% (8/26) of Cxcl12CE-tdTomato+ clones could be passaged over 4 generations, only 8.3% (4/48) of UbcCE-tdTomato+ clones could be passaged over the same generation (Fig. 2e, Supplementary Fig. 3b). These Cxcl12CE-tdTomato+ clones exhibited in vitro trilineage differentiation potential (i.e., adipocytes, osteoblasts, and chondrocytes, 12/12 clones, 100%, Fig. 3f), and differentiated into osteoblast-like cells depositing mineralized matrix upon transplantation into immunodeficient mice.
Fig. 1 Cxcl12-creER marks a quiescent subset of CXCL12+ LepR+ BMSCs. a Structure of Cxcl12-iCreER-bGHpA bacterial artificial chromosome (BAC) transgene, b–d Short-chase analysis of Cxcl12-creER+ cells, b Cxcl12GFP17; Cxcl12-creER; R26RtdTomato (pulsed at P21) distal femurs with growth plates on top. Gray: DAPI. Scale bar: 500 µm. n = 3 mice. c–d Immunostaining for endomucin (Emcn, c) and CD31 (d). Sn: sinusoids; At: arterioles. Arrows: sinusoid-attaching GFPhightdTomato+ cells. Gray: DAPI. Scale bar: 10 µm. n = 5 mice. e Percentage of cells attaching Emcn+ sinusoids among GFPhightdTomato+ (green) or GFPhightdTomato− (red) cells. n = 5 mice. f Flow cytometry analysis of CD45/Ter119/CD31− bone marrow cells. Blue lines: control cells. n = 5 mice. g Leptin receptor (LepR) expression based on flow cytometry analysis. Blue line: isotype control. n = 3 mice. h LepR staining. Gray: DAPI. (Center), DIC (metaphysis and endosteal zone). Scale bar: 20 µm. i Quantification based on histology. Percentage of BMSCs expressing Cxcl12-GFP (green dots), Cxcl12CE−tdTomato (red dots) and LepR (purple dots). n = 7 fields from four mice (central marrow and endosteal surface), n = 5 fields from 4 mice (metaphysis). j Percentage of CD45/Ter119/CD31− subpopulations in S/G2/M phase. n = 4 mice. k Intracellular Cxcl12 protein levels of CD45/Ter119/CD31− subpopulations. MFI: median fluorescence intensity. n = 5 mice. l Intracellular GFP protein levels of CD45/Ter119/CD31− subpopulations. MFI: median fluorescence intensity. n = 5 mice. m ColI (2.3 kb)-GFP; Cxcl12-creER; R26RtdTomato. n Left panel: trabecular bone. Right panel: endosteal space. Gray: DAPI. Scale bar: 20 µm. n = 4 mice. o Flow cytometry analysis of CD45/Ter119/CD31− cells harvested from ColI (2.3 kb)-GFP; Cxcl12-creER; R26RtdTomato at P28 (pulsed at P21) bone marrow. Blue lines: control cells. n = 4 mice. ***p < 0.0001, **p < 0.01, *p < 0.05, two-tailed, Mann–Whitney’s U test (e, l). Two-tailed, one-way ANOVA followed by Tukey’s post hoc test (f–k, m). All data are presented as mean ± s.d. Source data are provided as a Source Data file.

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Cell-fate analysis reveals dormancy of Cxcl12-creER+ BMSCs. We subsequently defined long-term cell fates of quiescent Cxcl12-creER+ BMSCs using Cxcl12-creER; R26RtdTomato mice carrying either ColI (2.3 kb)-GFP or Cxcl12GFP+ reporter. These mice were pulsed at P3, P21, and 8 weeks of age (hereafter, Cxcl12CE-P3, Cxcl12CE-P21, and Cxcl12CE-8 W cells, respectively). Cxcl12CE-P3 cells remained spatially restricted only in a far proximal portion of the femoral marrow space at 1 year of age (1 y) (Fig. 3a). Similarly, few Cxcl12CE-P21 cells migrated distally to contribute to newly formed marrow space beneath the growth plate toward 1 y (Fig. 3b, Supplementary Figs. 4a–g and 8a, b). By contrast, Cxcl12CE-8 W cells were distributed throughout the marrow space at 12 W and 1 y (Fig. 3c, Supplementary Fig. 4h), demonstrating that a tamoxifen pulse at the adult stage could label cells under the growth plate that were not labeled when tamoxifen was given at earlier stages (Fig. 3d). Therefore, these Cxcl12-creER+ BMSCs are dormant and remain in the original domain of the marrow space in growing bones, without producing new stromal cells in the newly formed domain of the marrow space in the metaphyseal region resulting from long-bone growth. These data also indicate that quiescent Cxcl12-creER+ BMSCs might be produced by other mesenchymal precursor cells during bone growth, such as type II collagen (Col2al)1+ cells residing in proximity to the growth plate or parathyroid hormone-related protein (PTHrP)1+ resting chondrocytes as demonstrated previously15,16.

Cxcl12-creER+ BMSCs marked by Cxcl12-creER remained in the marrow space predominantly as Cxcl12-GFPhigh reticular cells (Fig. 3e). Quantification of Cxcl12CE-8 W cells revealed that these cells did not change in number within the marrow space over time through 1 y 6 M (Fig. 3f). Importantly, Cxcl12-creER+ marked stromal cells, regardless of the time they were marked (i.e., Cxcl12CE-P3, Cxcl12CE-P21, Cxcl12CE-8 W, and Cxcl12CE-5 M cells), made virtually no contribution to cortical osteoblasts during the prolonged chase until 1 y 6 M (Fig. 3g–l, Supplementary Fig. 8a, b). Interestingly, these cells contributed to a fraction of trabecular osteoblasts/cytes (Fig. 3j–l). The fraction of Cxcl12CE-8 W cells among ColI (2.3 kb)-GFP+ trabecular osteoblasts/cytes increased progressively during the first 6 months of chase and reached the plateau thereafter (Fig. 3l red line). By contrast, essentially no Cxcl12CE-8 W cells contributed to cortical osteoblasts/cytes during the entire duration of chase (Fig. 3l, blue line). Therefore, quiescent Cxcl12-creER+ perisinusoidal BMSCs predominantly stay within the marrow space as Cxcl12+ stromal cells and do not migrate and differentiate into cortical bone osteoblasts in homeostasis.

Cortical bone regenerative responses of Cxcl12-creER+ BMSCs. Subsequently, we asked whether quiescent Cxcl12-creER+ BMSCs can participate in skeletal regeneration. The Cxcl12-creER line described here gives us the opportunity to experimentally interrogate their potential to participate in cortical bone regeneration, as quiescent Cxcl12-creER+ BMSCs do not normally differentiate into cortical bone osteoblasts. For this purpose, we utilized a drill-hole cortical injury model17 (Fig. 4a) that disrupts the femoral cortical bone and the endosteal surface to induce cortical bone regeneration through intramembranous ossification. We performed the surgery at 1 week after Cxcl12-creER; R26RtdTomato mice carrying either ColI (2.3 kb)-GFP or Cxcl12GFP+ reporter were pulsed at 6–10 weeks of age (Fig. 4b). After 2 days of injury, a substantial portion of Cxcl12CE−tdTomato+ cells adjacent to the injury site proliferated (EdU+ cells: 59.1 ± 10.8% of tdTomato+ cells, compared to 0.3 ± 0.7% in the intact site, n = 7), and invaded into the defect while maintaining Cxcl12-GFP expression (Fig. 4c–e). Cxcl12CE−tdTomato+ cells continued to increase at the injury site, and occupied the intertrabecular space of newly formed woven bones (hard callus) in the cortical bone after seven days of injury (Day 7, Fig. 4f); particularly those within the hard callus started to express ColI (2.3 kb)-GFP at this stage (Fig. 4g). After two weeks of injury, a majority of Cxcl12CE−tdTomato+ cells differentiated into ColI (2.3 kb)-GFP+ osteoblasts within the healing cortical bone (Day 14, Fig. 4h, i). After 8 weeks of injury, Cxcl12CE−tdTomato+ cells robustly contributed to osteocytes in the regenerated portion of the cortical bone (Day 56, Fig. 4j, k), which was not observed in the intact portion of the cortical bone (Cxcl12CE−tdTomato+ osteocytes: 0.1 ± 0.2% in Intact, 39.7 ± 1.9% in Injured, Fig. 4l, m). Therefore, quiescent Cxcl12-creER+ BMSCs in the marrow space can be recruited to a cortical defect in response to injury and differentiate into osteoblasts/cytes during cortical bone repair, unlike in homeostasis.

Multiple types of mesenchymal cells can participate in bone repair by becoming osteoblasts18. To address whether other osteoblast precursor cells can participate in cortical bone regeneration, we further took advantage of an osterix (Osx)-creER line19. We performed the surgery at 1 week after osteocalcin (Ocn)-GFP; Oss-creER; R26RtdTomato mice were pulsed at 6–10 weeks of age, and analyzed these mice after 8 weeks of injury. OssCE-tdTomato+ cells moderately contributed to
osteocytes in the regenerated portion of the cortical bone (Osx<sup>Cre</sup>-tdTomato<sup>+</sup> osteocytes: 12.3 ± 4.9%, Fig. 4m, n). These data suggest that Osx<sup>CreER</sup>+ cells might be one of other progenitor populations that contribute to approximately 60% of osteocytes in the regenerated portion that are not derived from Cxcl12-creER+ BMSCs.

As expected, Cxcl12-creER-marked stromal cells readily became marrow adipocytes. Cxcl12<sup>Cre</sup>-8 W and Cxcl12<sup>Cre</sup>-5 M cells spontaneously differentiated into Perillipin<sup>+</sup> marrow...
Fig. 2 Single-cell characterization of Cxcl12-creER+ BMSCs. a Single-cell RNA-seq analysis, UMAP-based visualization of major classes of Cxcl12-GFP+ cells (Clusters 0–8). Red dotted contour: reticular cells (Cluster 0). Center panels: tdTomato expression, feature plot (top), violin plot (Clusters 0–2) (bottom). Right panels: feature plots. Blue: high expression. n = 5858 cells merged from two biological replicates (Mouse #1: 2028 cells, Mouse #2: 3830 cells). b Split-dot-based visualization of representative gene expression in Cluster 0 (C-0, reticular cells) and Cluster-1 (C-1, pre-osteoblasts). Upper: Mouse #1, Lower: Mouse #2. Circle size: percentage of cells expressing a given gene in a given cluster (0–100%), color density: expression level of a given gene. c-f CFU-F assay. c Cxcl12CE-tdTomato+ (left) and UbcCE-tdTomato+ (right) bone marrow cells at P8 (pulsed at P21). Scale bar: 5 mm. d, e percentage of tdTomato+ colonies among total CFU-Fs, n = 4 (Cxcl12CE), n = 7 (UbcCE) mice. *p < 0.01, two-tailed, Mann–Whitney’s U test. Data are presented as mean ± s.d. d Survival curve of individual tdTomato+ clones over serial passages, n = 26 (Cxcl12CE), n = 48 (UbcCE) clones. Gehan–Breslow–Wilcoxon test. e, f In vitro lineage differentiation assay of Cxcl12CE-tdTomato+ clones (Passage 2–7). Upper panels: Adipogenic conditions. LipidTOX staining (left). Green: LipidTOX-Alexa488, red: tdTomato. Oil red O staining (Right). Center panels: Osteogenic conditions. Green: Col(2.3 kb)-GFP, red: tdTomato. Alizarin Red staining (right). Lower panel: Chondrogenic conditions, Alcian Blue staining. Insets: differentiation medium negative controls. Scale bar: 200 μm. n = 12 clones. Source data are provided as a Source Data file.

adipoctyes after the long chase, at 3 M and 1 y 6 M, respectively (Supplementary Fig. 5a). In addition, Cxcl12-creER+ reticular cells become marrow adipocytes in the presence of high-fat diet containing PPARγ agonist rosiglitazone (Rosi) (LipidTOX+tdTomato+ cell: Rosi, 39.3 ± 14.6 vs. Cont, 2.5 ± 1.0 cells per section, Supplementary Fig. 5b–e). A committed adipogenic subset of LepR+CXCL12+ cells in the marrow space is involved in bone fracture healing20. We, therefore, performed a tibial complete fracture experiment, a defect of which is repaired primarily through endochondral ossification. We performed the surgery at 1 week after ColI(2.3 kb)-GFP, Cxcl12-creER, R26R-tdTomato mice were pulsed at 6–10 weeks of age (Supplementary Fig. 5f). After 2 weeks of fracture, Cxcl12CE-tdTomato+ cells increased substantially within the fracture callus and became a number of Sox9+ cells (Supplementary Fig. 5g), indicating that Cxcl12-creER+ cells can differentiate into chondrocytes during fracture healing.

Injury-induced identity conversion of Cxcl12-creER+ BMSCs. We subsequently set out to define the mechanism underlying the regenerative capacity of Cxcl12-creER+ BMSCs. For this purpose, we investigated how injury induces changes in cell populations and their transcriptomes of quiescent Cxcl12-creER+ BMSCs by combined lineage-tracing and single cell RNA-seq analyses. As an injury model, we made use of a femoral bone marrow ablation model that mechanically removes a defined area of bone marrow using an endodontic instrument21 (Fig. 5a, b). This procedure induces direct differentiation of marrow stromal cells within the marrow cavity without involving the periosteum. The right femur underwent the surgery, while the left femur was untreated and used as an internal control (Fig 5a). Marrow ablation induced differentiation of Cxcl12CE-tdTomato+ cells into Col1α1(2.3 kb)-GFP+ osteoblasts within the marrow space (Fig. 5c–j, Supplementary Fig. 8a, b).

We interrogated the single-cell profile of lineage-marked Cxcl12CE-tdTomato+ cells from the ablated (ABL) and the contralateral control femur (CONT) individually after 7 days of marrow ablation, followed by integration of two datasets using Seurat/CCA22 (Fig. 6a, Supplementary Fig. 8c, e). Initial analysis revealed that a substantial fraction of cells belonged to the clusters without detectable tdTomato expression; these tdTomato− cells included myeloid cells, lymphocytes and erythroid cells (Supplementary Fig. 6a). tdTomato was predominantly expressed by mesenchymal cells expressing Cxcl12 or Col1α1, but also by a small number of endothelial cells. A graph-based clustering analysis of integrated mesenchymal Cxcl12CE-tdTomato+ cells revealed 12 clusters, including three major groups of Cxcl12+Adipoq+ reticular cells (Clusters 0–2) and small groups of Alpl+ osteoblastic cells (Clusters 4 and 6); a terminally differentiated osteoblast marker Ifitm5 was exclusively expressed by cells in Cluster 4 (Fig. 6b). Most notably, marrow ablation appeared to induce a shift in cell populations toward pre-osteoblasts and osteoblasts; ABL cells were more prominent in these osteoblastic clusters (Fig. 6c, yellow arrowheads). Concomitantly, cells in both reticular and pre-osteoblastic clusters appeared to undergo upregulation of osteoblast-signature genes in response to injury (Fig. 6d). Analysis of the second biological replicate showed a pattern similar to the first replicate (Supplementary Fig. 6a, b). In addition, a colony-forming assay revealed that marrow ablation induced a significant increase in Cxcl12CE-tdTomato+ CFU-Fs ex vivo (tdTomato+ colony: CONT: 1.4 ± 1.1% vs. ABL: 10.5 ± 6.5% of total CFU-Fs, Fig. 6e, f), consistent with a shift in cell populations toward pre-osteoblasts in the ablated marrow identified by the single cell RNA-seq analysis. Therefore, Cxcl12-creER+ BMSCs undergo a transcriptional change that drives them for an osteoblast-like state in response to injury, associated with an increase in colony-forming activities.

Cxcl12-creER+ BMSCs transform into skeletal stem-like cells. Further, we set out to define the functional importance of Cxcl12-creER+ BMSCs in cortical bone regeneration by inducible cell ablation experiments using a Rosa26-lsl-DTA (inducible diphtheria toxin fragment A) allele. Flow cytometry analysis of Cxcl12-creER; Rosa26-firesetdtTomato/− (tdT) and Cxcl12-creER; Rosa26-lsl-tdTomato/DTA (DTA) littermates at 16 weeks of age after being pulsed at 4, 6, and 8 weeks of age revealed a 6.8-fold reduction of tdTomato+ cells in DTA mice (tdTomato+ cells: tdT, 0.045 ± 0.004% vs. DTA, 0.007 ± 0.006% of CD45/Ter119/CD31+ve fraction, Fig. 7a, Supplementary Fig. 8a, n = 3 for Control, n = 6 for DTA mice), demonstrating the effectiveness of an iDTA-mediated cell ablation approach. Subsequently, we pulsed Rosa26-lsl-tdTomato/DTA (Control) and Cxcl12-creER; Rosa26-lsl-tdTomato/DTA (DTA) littermates with tamoxifen at 4 weeks of age, and performed the surgery at 1 week after the pulse. After 14 days of injury, bone volume and bone mineral density of the injured cortical area were significantly reduced in DTA mice (Fig. 7b, c). Therefore, quiescent Cxcl12-creER+ BMSCs are a functionally important contributor to cortical bone regeneration.

Further, we aimed to define how quiescent Cxcl12-creER+ BMSCs can be activated and embark on the differentiation trajectory into osteoblasts. For this purpose, we examined the single cell profile of lineage-marked Cxcl12CE-tdTomato+ cells from the ablated femur after 14 days of marrow ablation (Fig. 7d, Supplementary Fig. 8c, e). Initial analysis revealed again that a substantial fraction of cells (7074/9469 cells) belonged to the clusters without detectable tdTomato expression. A graph-based clustering analysis of mesenchymal Cxcl12CE-tdTomato+ cells revealed that pre-osteoblasts (preOBs) appeared as an intermediate state between Cxcl12+Adipoq+ perisinusoidal reticular cells and Bglap+ mature osteoblasts (Fig. 7e, Supplementary Fig. 7a–c). These intermediate-state cells expressed Itgav...
(encoding CD51) and Cd200 (CD200), but not Thy1 (CD90), Eng (CD105), Grem1 (Grem1), or Ly6a (Sca1), indicating that these cells might overlap with previously reported mouse SSCs (mSSCs)\textsuperscript{23}, but not with osteo-chondro-reticular stem cells\textsuperscript{24} (Fig. 7f). We further constructed an unsupervised single-cell trajectory using Monocle (Supplementary Fig. 7d). This analysis predicted a continuous lineage path without a branch, and empirically identified pseudotime-dependent genes that were highly expressed in populations corresponding to perisinusoidal reticular cells, pre-osteoblasts and mature osteoblasts (Fig. 7g, h). Functional annotation of 970 pseudotime-dependent differentially expressed genes using DAVID revealed significant enrichment of Gene Ontology terms related to Wnt signaling (3 out of top 7 most significant terms, Wnt signaling pathway [GO:0016055], negative regulation of Wnt [GO:0030178] and canonical Wnt [GO:0090090] signaling pathway) (Fig. 7i). In fact,
genes encoding soluble Wnt antagonists including Sfrp1, Sfrp2 and Sfrp4 were enriched in perisinusoidal reticular cells, whereas Ctnnb1, a key component of Wnt/β-catenin signaling, was enriched in pre-osteoblasts. Therefore, quiescent Cxcl12-creER+ BMSCs appear to undergo activation of canonical Wnt signaling during their transition into pre-osteoblasts.

Wnt-mediated transformation orchestrates bone regeneration. To test the hypothesis that canonical Wnt signaling plays an important role in this transition, we conditionally deleted β-catenin (Ctnnb) in Cxcl12-creER+ BMSCs during cortical bone regeneration, using Cxcl12-creER; Ctnnbfl/+; R26RDTomato (Control), Cxcl12-creER; Ctnnbfl/fl; R26RDTomato (βCat cHet) and Cxcl12-creER; Ctnnbfl/fl; R26RDTomato (βCat cKO) littermates. After 14 days of cortical bone injury, bone volume and bone mineral density of the injured cortical area were significantly reduced in βCat cKO mice compared with Control or βCat cHet mice, associated with reduction in alkaline phosphatase (ALP) cells (Fig. 8a, b). The reduction of bone volume in βCat cKO mice was comparable to that of DTA mice, indicating that canonical Wnt signaling plays essential functional roles in Cxcl12-creER+ BMSCs’ differentiation trajectory toward osteoblasts.

Transcription factors Sox9 and Runx2 cooperatively regulate commitment to the osteoblast lineage in a manner regulated by canonical Wnt signaling during skeletal development25,26. However, our analysis showed that Sox9 expression was unaltered, while Runx2 expression was not consistently altered in response to injury (Fig. 6d). To test whether Sox9 and Runx2 play roles in cell identity conversion, we conditionally deleted Sox9 or Runx2 in quiescent Cxcl12-creER+ BMSCs during cortical bone regeneration, using Cxcl12-creER and floxed alleles for Sox9 and Runx2. After 14 days of cortical bone injury, bone volume and bone mineral density of the injured cortical area was not altered in Sox9 cKO mice or in Runx2 cKO mice (Fig. 8c), indicating that their transition into pre-osteoblasts is not regulated by Sox9 or Runx2.

Further, to address whether high levels of β-catenin can inhibit osteoblast differentiation of Cxcl12-creER+ BMSCs during cortical bone regeneration, we conditionally induced haploinsufficiency of adenomatous polyposis coli (Apc), which is a critical component of the β-catenin degradation complex, during cortical bone regeneration using Cxcl12-creER; Apcfl/+; R26RDTomato (Control) and Cxcl12-creER; Apcfl/fl; R26RDTomato (Apc cHet) littermates. After 7 days of cortical bone injury, there was no significant change in bone volume and bone mineral density of the injured cortical area in Apc cHet mice (Fig. 8d). Therefore, a high level of β-catenin in Cxcl12-creER+ BMSCs due to APC haploinsufficiency does not inhibit their osteoblast differentiation during cortical bone regeneration. A limitation of this approach is that it provides a different level of β-catenin stabilization than removing the phosphorylation sites encoded by exon 3.

Canonical Wnt signaling plays disparate roles in different phases of bone fracture repair27. To determine whether Cxcl12-creER+ cells are more or less sensitive to β-catenin than other fracture repair sources, we conditionally deleted Ctnnb in osteoblast precursor cells during cortical regeneration, using Ctnnbfl/fl; R26RDTomato (Control) and Dlx5-creER; Ctnnbfl/fl; R26RDTomato (Dlx5-βCat cKO) littermates. After 14 days of cortical bone injury, bone volume and bone mineral density of the injured cortical area were significantly reduced Dlx5-βCat cKO mice compared with Control mice (Fig. 8e, f). Therefore, Cxcl12-creER+ BMSCs are equally as sensitive to β-catenin as Dlx5-creER+ osteoblast precursor cells.

Taken together, our findings provide functional evidence that quiescent Cxcl12-creER+ BMSCs orchestrates cortical bone regeneration in a unique manner mediated by canonical Wnt signaling independent of Sox9 or Runx2 function.

Discussion

Here, we identified that quiescent Cxcl12-creER+ BMSCs can convert their identity into a precursor cell state similar to SSCs during injury responses in a manner mediated by canonical Wnt signaling, and substantially contribute to skeletal regeneration (see Fig. 9a–c). Importantly, these Cxcl12-creER+ BMSCs show little colony-forming activities and do not contribute to cortical bone osteoblasts in homeostasis or new CXCL12+ cells in growing marrow space, therefore representing a specific quiescent subpopulation of LepR+CXCL12+ BMSCs marked by LepRcre or Ebf3-creER79. LepR+CXCL12+ cells that contribute to cortical bone osteoblasts in physiological conditions are within the majority of perisinusoidal cells that are not marked by Cxcl12-creER. Injury-induced cues drive these Cxcl12-creER+ BMSCs into a reticular-osteoblast hybrid state associated with global upregulation of osteoblast-signature genes. Our findings support the theory that canonical Wnt-mediated cellular plasticity underpins the remarkable regenerative capacity of CXCL12+ BMSCs. Canonical Wnt signaling is widely known as the central regulator of osteogenesis, particularly during early skeletal development25,26. In adult bones, canonical Wnt signaling prevents a cell fate shift of preosteoblasts to adipocytes28 and inhibits excessive osteoclastogenesis through regulating mature osteoblasts29. In addition, liposomal delivery of Wnt proteins can promote bone regeneration30. Our findings highlight a mechanism that canonical Wnt signaling facilitates osteogenesis by
transforming CXCL12⁺ BMSCs into osteoblast precursor cells particularly under regenerative conditions. CXCL12⁺ BMSCs are equally as sensitive to deficiency in canonical Wnt signaling as other osteoblast precursor cells. This Wnt-mediated mechanism appears to be unique because, unlike in skeletal development, it does not mediate Sox9 or Runx2 function.

The remarkable regenerative potential of bones is generally attributed to rare SSCs. Our findings raise an intriguing possibility that the previously defined SSCs²³ can be generated through
Fig. 4 Cortical bone regenerative responses of Cxcl12-creER<sup>T2</sup> BMSCs. (a–n) Fates of Cxcl12-creER<sup>T2</sup> cells or Osx-creER<sup>T2</sup> cells during cortical bone regeneration after drill-hole injury. a Experimental schemes for drill-hole cortical injury model. b Timeline for tamoxifen injection, drill-hole injury and analysis. Cxcl12<sup>CreT2</sup>; Cxcl12-creER, R26R<sup>TdtTomato</sup> (c, f) or Col1(2.3 kb)-GFP; Cxcl12-creER, R26R<sup>TdtTomato</sup> (g–k) femur diaphyseal cortical bones (pulsed at 7 days before surgery) with periosteum on top. CB cortical bone, BM bone marrow. Gray: DIC. n = 5 mice per each group. c, d Injured area at 2 days after injury, femurs with EdU administration shortly before analysis. Scale bar: 200 µm (c), 50 µm (d). e Quantification of EdU<sup>+</sup>tdTomato<sup>+</sup> cells. n = 6 (Intact), n = 7 (Injured). EdU was administered twice (6 and 3 h) prior to analysis. **p < 0.01, two-tailed Mann-Whitney’s U test. f, g Injured area at 7 days after injury, femurs with EdU administration shortly before analysis. Scale bar: 200 µm (f), 20 µm (g). h, i Injured area at 14 days after injury, femurs with EdU administration shortly before analysis. Scale bar: 200 µm (h), 20 µm (i). j–l Injured cortical bone at 56 days after injury. Scale bar: 200 µm (h), 20 µm (i). m Number of tdTomato<sup>+</sup> cells within intact and injured area of Cxcl12-creER, R26R<sup>TdtTomato</sup> cortical bone (blue and red dots, respectively) or injured area of Osx-creER, R26R<sup>TdtTomato</sup> cortical bone (orange dots). n = 5 mice per group. *p < 0.01, two-tailed Mann-Whitney’s U test. n Injured cortical bone at 56 days after injury, Ocn-GFP; Osx-creER; R26R<sup>TdtTomato</sup> femurs. All data are presented as mean ± s.d. Source data are provided as a Source Data file.

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Fig. 5 Cxcl12-creER<sup>T2</sup> BMSCs respond to bone marrow ablation-induced osteogenesis. a–c Experimental schemes and procedures of bone marrow ablation model. a Right femurs were operated, while left femurs were unoperated and used as internal control. (b, i–vi): Step-by-step demonstration of surgical procedures. c Timeline for tamoxifen injection, ablation surgery and analysis. d–j Col1(2.3 kb)-GFP; Cxcl12-creER, R26R<sup>TdtTomato</sup> distal femur bone marrow (pulsed at 7 days before surgery) with growth plates on top. Three days (d), 7 days (e), and 14 days after surgery (f, h). Ablated area, boxed area in (f). Scale bar: 50 µm. g Contralateral control side. Gray: DIC. Scale bar: 500 µm. n = 4 (Day 3), n = 3 (Day 7), n = 5 (Day 14), n = 3 (Control) mice. i Flow cytometry analysis of CD45/Ter119/CD31<sup>−</sup> bone marrow cells. Lower panels: fold change of total tdTomato<sup>+</sup> cells (left) or Col1-GFP<sup>+</sup> tdTomato<sup>+</sup> cells (right) in ablated side over contralateral control side. Scale bar: 20 µm. n = 6 mice. *p < 0.01, two-tailed Mann-Whitney’s U test. Data are presented as mean ± s.d. j Regenerated cartilaginous tissue adjacent to growth plate. Gray: DIC. Source data are provided as a Source Data file.
transformation of mature stromal cells. This is reminiscent of a model proposed in the adult liver and pancreas, both of which possess high regenerative capacity despite slow cell turnover; these organs primarily depend on mature cells for tissue maintenance in homeostasis and regeneration in response to injury. We assume that mature stromal cells abundantly available in the milieu provide the primary source of cells executing bone repair under markedly slow cell turnover in the adult bone, in conjunction with a small number of resident osteoblast precursor cells. These highly malleable, long-living mature stromal cells readily available throughout the lifespan represent an ideal cellular source for tissue regeneration. Further mechanistic studies are needed to exploit these cells better for future regenerative medicine.

Methods
Generation of Cxcl12-creER BAC transgenic mice. Cxcl12-creERT2-bGHpA transgenic mice were generated by pronuclear injection of a modified BAC clone RP23-22F6 (Children’s Hospital Oakland Research Institute, Oakland, CA) containing 74 kb upstream and 121 kb downstream genomic sequences of the Cxcl12 gene. Hybrid primers (Fw: ACTTTCACTCTCGGTCCACCTCGGTGTCCTC, L: Tamoxifen (−7 day): BM ablation (Day 0) – 6 weeks; [Day 7] CONT (L) ABL (R), Cell harvest (Day 7)

**Fig. 6** Injury-induced identity conversion of Cxcl12-creER+ BMSCs. a–d Single-cell RNA-seq analyses of lineage-traced Cxcl12-creER+ cells. a Cxcl12CE-tdTomato+ cells were isolated from contralateral (CONT) and ablated (ABL) femurs separately at 7 days after marrow ablation. Two datasets were integrated by Seurat/CCA. b, c UMAP-based visualization of major classes of mesenchymal Cxcl12CE-tdTomato+ cells (Cluster 0–11). Left panel, red dotted contour: reticular (Clusters 0–2). Lower panels: feature plots. Blue: high expression. Right panel: cells colored by conditions (CONT, ABL). Biological replicate 1, n = 2939 cells (CONT: 2066 cells, ABL: 873 cells), pooled from n = 7 mice. d Split-dot-based visualization of representative gene expression. Clusters 0 and 1 (reticular) and Cluster 6 (pre-osteoblast) shown for reticular-signature genes (Cxcl12, Adipoq) and osteoblast-signature genes (Col1a1, Bglap, Spp1, Alpl, Postn, Tnc, Pth1r, Sox9, Runx2, Sp7, Atf4). *p < 0.0001, Wilcoxon rank sum test. Circle size: percentage of cells expressing a given gene in a given cluster (0–100%). Color density: expression level of a given gene. e, f CFU-F assay of Cxcl12CE-tdTomato+ bone marrow cells, harvested from contralateral (CONT, left) and ablated (ABL, right) femurs at Day 7 (pulsed at Day−7). Scale bars: 5 mm. g Percentage of tdTomato+ colonies among total CFU-Fs. n = 5 mice per group. *p < 0.05, two-tailed, Mann-Whitney’s U test. Data are presented as mean ± s.d. Source data are provided as a Source Data file.
TTGCTGTCCAGCTCTGCAGCCTCCGGCGCGCCCTCCCGCCCACGCCACCATGGTGCCCAAGAAGAAGAG, Rv: CGGGCCCGGCCGCGGATTGCACTCACGTCACTGATGCAGAGCGCGGCCAGCACCAGGGCCAGCACGGCGACGACCTCTATACGAAGTTATAAGCTT) containing 77 bp homology arms specific to the first exon of the Cxcl12 gene were used to polymerase chain reaction (PCR)-amplify the targeting construct from the plasmid containing the iCreERT2-bGHpA sequence. The PCR product was gel-purified prior to electroporation into Escherichia coli strain SW105 containing RP23-22F6. Recombinants were selected on bacterial plates containing kanamycin and PCR-screened for homologous recombination events, followed by pulsed-field gel electrophoresis analysis (CHEF-DRIII, Bio-rad) to confirm no gross rearrangement of the targeted BAC clones. The sequence of the BAC-insert junction was further validated based on PCR. The
Neor cassette was removed from correctly targeted BAC doses by -arabinose induction of tpe recombine, and lox sites (loxP and lox511) in the BAC plasmid were replaced with AmpR and NeoR cassettes by recombining. The modified BAC clone was verified by pulsed-field gel electrophoresis analysis, and purified by NucleoBond Xtra BAC purification kit (Clontech). The BAC clone was linearized by BsaW1 digest, and a 207 kb fragment containing ckl12-CreR3′ was separated by size-exclusion chromatography (Sepharose CL-4B). The fragment eluted from the column was collected in multiple fractions in polyamine micro-injection buffer to protect the DNA from shearing, followed by pulsed-field gel electrophoresis (PFGE) concentration of the BAC construct was verified on Nanodrop 2000 spectrophotometer. The BAC clone was microinjected into B6SJL-F1 fertilized eggs at a concentration of 0.25-0.5 ng/µl. Genomic DNA from potential founders were genotyped with PCR primers (320: TTTCACTCTCG NAnAc200 spectrophotometer. The BAC clone was microinjected into bone marrow stromal cells when bred to an tamoxifen-ethanol-oil mixture was incubated at 60°C in a chemical hood until the cell trajectory analysis by Monocle.

Surgery. Mice were anesthetized through a nose cone, in which 1.5-2% isoflurane was constantly provided with oxygen through a vaporizer.

For drill-hole injury, left femurs were operated, while right femurs were untreated and used as an internal control. An incision was made in the lateral skin of the mid-femur. After splitting the muscle, the periosteum was separated to expose the femur cantor. A drill-hole injury was made onto the diaphysis by a Micro-Drill (Stoelting) with a 0.9 mm diameter stainless steel burr (Fine Science Tools). The peristem of the surgical field was scapped off with a scapel prior to the procedure.

For bone marrow ablation surgery, right femurs were operated, while left femurs were untreated and used as an internal control. After skin incision, cruciate ligaments of the knee were carefully separated using a dental excavator, and a hole was made in the intercondylar region of femurs using a 26-1/2 gauge needle (BD395957). The endodontic instruments (K-file, #35, 40, 45, and 50) (GC, Tokyo, Japan) were used in a stepwise manner to remove a cylindrical area of the marrow space of the cortical field with saline, and the incision line was cultured.

For tibial complete fracture healing model, legs were aseptically prepared using Chlorhexidine scrub, once mice were anesthetized. A small incision was made on the anterior-medial surface of the proximal tibial metaphysis, exposing the knee joint and patellar tendon. Using a small gauge needle, an entry hole was made distally through the proximal femur and recharged with saline. A narrow stainless steel wire was placed and extended through the intramedullary canal until contact at the distal end. The pin was trimmed flush with the proximal tibial condyles, and skin and soft tissue are closed with suture. While remaining under anesthesia, animals were positioned under a custom-built guillotine apparatus such that upon impact, a transverse fracture was made at the mid-distal tibial diaphysis. Tibias were gently stabilized with a small length of medical tape to provide support and biomechanical stability for immediate ambulation. Animals were subsequently recovered and returned to housing cages.

Histology and immunohistochemistry. Samples were fixed in 4% paraformaldehyde for a proper period, typically ranging from 3 h to overnight at 4°C, then decalcified in 15% ethylenediaminetetraacetic acid for a proper period, typically ranging from 3 h to 14 days. Decalcified samples were cryoprotected in 30% sucrose/PBS solutions and then in 30% sucrose/PBS:OCT (1:1) solutions, each at least overnight at 4°C. Samples were embedded in an OCT compound (Tissue-Tek, Sakura) and cryosectioned at 14 µm using a cryostat (Leica CM1830) and adhered to positively charged glass slides (Fisherbrand ColorFrost Plus). Sections were postfixed in 4% paraformaldehyde for 15 min at room temperature. For immunohistochemistry, sections were permeabilized with 0.25% Triton X-100 in PBS for 30 min, blocked with 3% bovine serum albumin/TBST for 30 min. and incubated with rat anti-endothelin (Emcn) monoclonal antibody (1:100, Santa Cruz Biotechnology, sc-65495), rat anti-CD31 monoclonal antibody (1:100, Bio-Rad, MCA2388), goat anti-leptin receptor (LepR) polyclonal antibody (1:100, R&D, AF497), goat anti-osteopontin (OPN) polyclonal antibody (1:500, R&D, AF808), goat anti-ALP polyclonal antibody (1:100, R&D, AF2910), rabbit anti-SOX9 polyclonal antibody (1:500, EMD-Millpore, AB5535), rabbit anti-perilipin A/B polyclonal antibody (1:500, Sigma, S1873) or rabbit anti-PPAR gamma (PPARy) polyclonal antibody (1:100, Invitrogen, MA5-14889) overnight at 4°C, and then washed twice with PBS. Primary antibodies were visualized using DAPI (4′,6-diamidino-2-phenylindole, 5 µg/ml, Invitrogen) or Alexa Fluor 633-conjugated goat anti-rat IgG (A31573) (1:400, Invitrogen) for 3 h at room temperature. For EdU assay, sections were incubated with Alexa Fluor 647-azide (Invitrogen, A10277) for 30 min at 45°C using Click-iT Imaging Kit (Invitrogen, C10377). Sections were stained by DAPI (4′,6-diamidino-2-phenylindole) and 4% paraformaldehyde (0.5% Triton X-100, 0.1% sodium nitro blue tetrazolium stain) to stain nuclei prior to imaging. For lipid staining, cryosections were immediately cover-slipped with LipidTOX Deep Red (1:200, Invitrogen, H34477)

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**Table**

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<td>Cassette removed from correctly targeted BAC doses by -arabinose induction of tpe recombine, and lox sites (loxP and lox511) in the BAC plasmid were replaced with AmpR and NeoR cassettes by recombining. The modified BAC clone was verified by pulsed-field gel electrophoresis analysis, and purified by NucleoBond Xtra BAC purification kit (Clontech). The BAC clone was linearized by BsaW1 digest, and a 207 kb fragment containing ckl12-CreR3′ was separated by size-exclusion chromatography (Sepharose CL-4B). The fragmen eluted from the column was collected in multiple fractions in polyamine micro-injection buffer to protect the DNA from shearing, followed by pulsed-field gel electrophoresis (PFGE) concentration of the BAC construct was verified on Nanodrop 2000 spectrophotometer. The BAC clone was microinjected into B6SJL-F1 fertilized eggs at a concentration of 0.25-0.5 ng/µl. Genomic DNA from potential founders were genotyped with PCR primers (320: TTTCACTCTCG NAnAc200 spectrophotometer. The BAC clone was microinjected into bone marrow stromal cells when bred to an tamoxifen-ethanol-oil mixture was incubated at 60°C in a chemical hood until the cell trajectory analysis by Monocle.</td>
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**Fig. 8 Wnt-mediated transformation orchestrates bone regeneration.**

**a.** Functional analysis of Wnt/β-Catenin signaling in Cxcl12-creER<sup>+</sup> cells during cortical bone regeneration. Cxcl12-creER; Ctnnb<sup>fl/fl</sup>; R26R<sup>TdTomato</sup> (Control, left), Cxcl12-creER; Ctnnb<sup>fl/+</sup>; R26R<sup>TdTomato</sup> (βcat cKO, right) femur diaphyseal cortical bones, 14 days after cortical drill-hole surgery (pulsed at 7 days before surgery). **b.** Cortical bone injured areas. Upper panels: alkaline phosphatase (ALP) staining. Gray: DIC. Scale bar: 200 µm. Lower panels: 3D-reconstructed bone mineral density (BMD, right) of the injured area, n = 4 (Control), n = 8 (βcat cKO) mice. Functional analysis of Sox9 and Runx2 in Cxcl12-creER<sup>+</sup> cells during cortical bone regeneration. Cxcl12-creER; Sox9<sup>fl/+</sup> or Runx2<sup>fl/+</sup>; R26R<sup>TdTomato</sup> (Control, left), Cxcl12-creER; Sox9<sup>fl/+</sup> or Runx2<sup>fl/+</sup>; R26R<sup>TdTomato</sup> (Sox9 cKO or Runx2 cKO, center) and Cxcl12-creER; Sox9<sup>cat+cKO</sup>; Runx2<sup>fl/+</sup>; R26R<sup>TdTomato</sup> (Sox9 cKO or Runx2 cKO, right) femur diaphyseal cortical bones, 14 days after cortical drill-hole surgery (pulsed at 7 days before surgery). BV/TV (left) and BMD (mg/cm<sup>3</sup>) (right) of the injured area, n = 7 (Sox9 Control), n = 4 (Sox9 cKO or Runx2 cKO, cKO) mice. n = 6 (Runx2 Control, cKO), n = 8 (Runx2 cKO) mice. **d.** Functional analysis of Apc in Cxcl12-creER<sup>+</sup> cells during cortical bone regeneration. Cxcl12-creER; Apc<sup>fl/+</sup>; R26R<sup>TdTomato</sup> (Control, left), Cxcl12-creER; Apc<sup>fl/-</sup>; R26R<sup>TdTomato</sup> (Apc cKO, right) femur diaphyseal cortical bones, 7 days after cortical drill-hole surgery (pulsed at 7 days before surgery). BV/TV (left) and BMD (right) of the injured area, n = 6 (Control), n = 4 (Apc cKO) mice. **f.** Functional analysis of Wnt/β-Catenin signaling in Dlx5-creER<sup>+</sup> cells during cortical bone regeneration. Ctnnb<sup>fl/+</sup>; R26R<sup>TdTomato</sup> (Control, left), Dlx5-creER; Ctnnb<sup>fl/+</sup>; R26R<sup>TdTomato</sup> (Dlx5-βcat cKO, right) femur diaphyseal cortical bones, 14 days after cortical drill-hole surgery (pulsed at 7 days before surgery). **3D-µCT.** Lower panels: BV/TV (left) and BMD (right) of the injured area. Scale bar: 1mm. n = 6 mice per group. ****p < 0.0001, ***p < 0.001, **p < 0.05, two-tailed, one-way ANOVA followed by Tukey’s post hoc test (b, c). Two-tailed, Mann-Whitney’s U test (d, f). All data are presented as mean ± s.d. Source data are provided as a Source Data file.
and 5 µg/ml DAPI solution and incubated for 2 h at 43 °C. Stained samples were mounted in TBS with No. 1.5 coverslips (Fisher).

**Imaging.** Images were captured by an automated inverted fluorescence microscope with a structured illumination system (Zeiss Axio Observer Z1 with ApoTome.2 system) and Zen 2 (blue edition) software. The filter settings used were: FL Filter Set 34 (Ex. 390/22, Em. 460/50 nm), Set 38 HE (Ex. 470/40, Em. 525/50 nm), Set 43 HE (Ex. 550/25, Em. 605/70 nm), Set 50 (Ex. 640/30, Em. 690/50 nm). The objectives used were: Fluar 2.5×/0.12, EC Plan-Neofluar 5×/0.16, Plan-Apochromat 10×/0.45, EC Plan-Neofluar 20×/0.50, EC Plan-Neofluar 40×/0.75, Plan-Apochromat 63×/1.40. Images were typically tile-scanned with a motorized stage, Z-stacked and reconstructed by a maximum intensity projection function. Differential interference contrast was used for objectives higher than 10×. Representative images of at least three independent biological samples are shown in the figures. Quantification of cells on sections was performed using NIH Image J software.

**Cell dissociation.** Soft tissues and epiphyses were carefully removed from dissected femurs. After removing distal epiphyseal growth plates and cutting off proximal ends, femurs were cut roughly and incubated with 2 Wunsch units of Liberase™ (Sigma/Roche 5401127001) and 1 mg of Pronase (Sigma/Roche 10165921001) in 2 ml Ca²⁺, Mg²⁺-free Hank’s Balanced Salt Solution (HBSS, Sigma H6648) at...
pellet the cells. The pellet was cultured in the differentiation medium with exchanges into fresh media every 3–4 days for up to 3 weeks, each time with centrifugation at 150g for 5 min at room temperature to pellet the cells. Cell pellets were fixed with 70% ethanol for 5 min. and stained for Alcian-Blue Staining Solution (Millipore TMS-010-C) for 30 min. To induce osteoblast differentiation, cells were plated on a 48-well plate and cultured with αMEM with GlutaMAX supplement (Gibco 11965–092, 10 mM β–glycerophosphate (Sigma C4281) and 30 µg/mL ascorbic acid (Sigma A5960)) with exchanges into fresh media every 3–4 days for up to 3 weeks. Cells were fixed with 4% paraformaldehyde for 5 min and stained for 2% Alizarin Red S (Sigma A1883) for 30 min. To induce adipocyte differentiation, cells were cultured on a 48-well plate and cultured with αMEM with GlutaMAX supplement with 10% FBS containing 1 µM dexamethasone (Sigma D9802), 10 mM β–glycerophosphate (Sigma C4281) and 30 µg/mL ascorbic acid (Sigma A5960) with exchanges into fresh media every 3–4 days for up to 3 weeks. Cells were fixed with 4% paraformaldehyde for 5 min and stained for 1% Oil Red O (Sigma O0625).

Subcutaneous transplantation of cultured cells. NOD scid gamma (NSG) (JAX05557) mice were used as recipients for cell transplantation. Mice were anesthetized through a nose cone, in which 1.5–2% isoflurane is constantly provided with oxygen through a vaporizer. Fur was removed with clippers and depilatory cream in a clean area. Matrigel (Corning) was mixed with 108–1010 cells (cultured Cxcl12+CreER+ stromal cells, Passage 10 or higher), and loaded into an insulin syringe (BD-Lo-Dose U-100 Insulin Syringes, BD329461). The cell-matrix mixture was injected subcutaneously into a designated spot in the back skin or the calvaria.

Three-dimensional micro-computed tomography analysis. Femurs were dissected and scanned by micro-computed tomography (microCT) (Skyscan 1172; Bruker microCT, GE Healthcare Pre-Clinical Imaging, London, ON, Canada). Scans were reconstructed at 18 µm isotropic voxel size and analyzed using Microview v2.2 (GE Healthcare, London, Ontario, Canada). For analysis of the healing of the 0.9 mm drill hole defect in the diaphyseal femur, images were re-oriented to align the direction of drilling with the y-axis. A 0.7 mm diameter cylindrical region of interest (ROI) was centered immediately inside the cortex, and in the cortical gap with a fixed thickness of 0.2 mm. This ROI typically spanned the cortical thickness from the endosteal-to-periosteal surface. Regenerated sites were assessed for bone volume fraction using a threshold of 2300 Hounsfield Units (HU). A common threshold was calculated across all samples by using the auto-threshold tool of microView for each specimen. 3D images were reconstructed using CT-Analyzer (Bruker microCT).

Statistical analysis. Results are presented as mean values ± S.D. Statistical evaluation was conducted using the Mann–Whitney’s U test or one-way ANOVA. A p value <0.05 was considered significant. No statistical method was used to predetermine the sample size. The sample size was determined on the basis of previous literature and our previous experience to give sufficient standard deviations of the mean so as not to miss a biologically important difference between groups. The experiments were not randomized. All of the available mice of the desired genotypes were used for experiments. The investigators were not blinded during experiments and outcome assessment. One femur from each mouse was arbitrarily selected for analysis. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. The single-cell RNA-seq data presented herein was deposited in the National Center for Biotechnology Information (NCBI)’s Gene Expression Omnibus (GEO), and are accessible through GEO Series accession numbers GSE136970, GSE136973, and GSE136979. The source data underlying all Figures and Supplementary Figures are provided as a Source Data file.

Code availability
The custom codes utilized for single cell RNA-seq analysis of this study are publicly available from the following websites: Seurat 3.0 R package (https://satijalab.org/seurat/) and Monocle 2 R package (http://cole-trapnell-lab.github.io/monocle-release/).

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Author contributions


Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to N.O.

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