Discovery of a periosteal stem cell mediating intramembranous bone formation

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Bone consists of separate inner endosteal and outer periosteal compartments, each with distinct contributions to bone physiology and each maintaining separate pools of cells owing to physical separation by the bone cortex. The skeletal stem cell that gives rise to endosteal osteoblasts has been extensively studied; however, the identity of periosteal stem cells remains unclear4-7. Here we identify a periosteal stem cell (PSC) that is present in the long bones and calvarium of mice, displays clonal multipotency and self-renewal, and sits at the apex of a differentiation hierarchy. Single-cell and bulk transcriptional profiling show that PSCs display transcriptional signatures that are distinct from those of other skeletal stem cells and mature mesenchymal cells. Whereas other skeletal stem cells form bone via an internal cartilage template using the endochondral pathway3, PSCs form bone via a direct intramembranous route, providing a cellular basis for the divergence between intramembranous versus endochondral developmental pathways. However, there is plasticity in this division, as PSCs acquire endochondral bone formation capacity in response to injury. Genetic blockade of the ability of PSCs to give rise to bone-forming osteoblasts results in selective impairments in cortical bone architecture and defects in fracture healing. A cell analogous to mouse PSCs is present in the human periosteum, raising the possibility that PSCs are attractive targets for drug and cellular therapy for skeletal disorders. The identification of PSCs provides evidence that bone contains multiple pools of stem cells, each with distinct physiologic functions.

A major limitation to identifying periosteal stem cells has been the lack of genetic markers that discriminate between periosteal and endosteal mesenchyme. While studying the specificity of skeletal targeting cre strains, we observed that cathepsin K–Cre (Ctskcre) labels the periosteal mesenchyme. In Ctskcre Rosa26ERT2tmG reporter mice5, in which Ctskcre cells and their progeny (hereafter CTSK–mGFP cells) express membrane-bound GFP (mGFP), labelling of the periosteal mesenchyme was observed as early as embryonic day 14.5 (E14.5) (Extended Data Fig. 1a–e). At postnatal day 10, CTSK–mGFP cells were observed in the periosteal mesenchyme and the endosteal marrow compartment, although nearly all of the endosteal cells were morphologically consistent with osteoclasts (Fig. 1a, Extended Data Fig. 1f). A negligible number of osteocysts were CTSK–mGFP+ (Extended Data Fig. 1g). Flow cytometry of endosteal cells and co-staining for tartrate-resistant acid phosphatase (TRAP) confirmed that endosteal CTSK–mGFP cells were osteoclasts (Fig. 1b, c). Conversely, the majority of CTSK–mGFP cells in the periosteum were CD45-TER119-CD31- (hereafter Lin-) mesenchymal cells (Fig. 1c). Periosteal CTSK–mGFP cells include periosteal osteoblasts, as shown by expression of type I collagen, Runx2, alkaline phosphatase (ALPL) and osteocalcin (Fig. 1d, Extended Data Fig. 1j). Therefore, within the mesenchymal compartment, Ctskcre selectively labels the periosteum (Fig. 1c).

This labelling suggested that a periosteal stem cell exists within the mesenchymal CTSK–mGFP+ population (Fig. 1a–c). To test this, we fractionated CTSK–mGFP mesenchymal cells using multi-colour flow cytometry (Fig. 1f–j, Extended Data Fig. 1i). We observed three populations among CTSK–mGFP cells lacking THY1.2 and 6C3, all of which were CD49b-CD51b-CD200-CD105+ periosteal mesenchymal stem cells (PSCs), CD200-CD105+ periosteal progenitor 1 (PP1) cells, and CD105+CD200+ variable periosteal progenitor 2 (PP2) cells (Fig. 1f, g, Extended Data Fig. 2a–c). Immunostaining confirmed the presence of CD200+ CTSK–mGFP cells in the periosteum and also identified subsets of CTSK–mGFP cells expressing gremlin1 and nestin (Fig. 1e, Extended Data Fig. 1b, e, k). Consistent with the restriction of haematopoiesis to the endosteal compartment, CTSK–mGFP+ status, and expression of LEPR, CD41 and CD44 markers that are present in mesenchymal cells and have the ability to support haematopoiesis3,6,9,10—are mutually exclusive (Fig. 1h–l, Extended Data Fig. 2e–g). During embryonic development, periosteal CD200+C TSK–mGFP cells consistent with PSCs were first observed at E14.5, concurrent with the onset of skeletal mineralization, and were distinct from CD200+ cells present within the chondroepiphysis (Extended Data Fig. 1a–e).

PSCs display the stem cell properties of clonal multipotency, self-renewal and the ability to give rise to the entire range of CTSK–mGFP cells. Serial mesensphere formation is an in vitro proxy for self-renewal1, and only PSCs possessed the capacity to form tertiary mesospheres, retaining CD200 through this process (Fig. 2a–c). To determine which periosteal population sits at the apex of the CTSK–mGFP differentiation hierarchy, cell populations isolated by fluorescence-activated cell sorting (FACS) were cultured for 15 days, and subsequently analysed by flow cytometry; these PSCs differentiated into PP1 and PP2 cells in addition to THY1.2+ and 6C3+ cells (Fig. 2d). By contrast, PP1s or PP2s did not produce PSCs in culture (Extended Data Fig. 2i). Additionally, PSCs demonstrated in vitro clonal multipotency for differentiation into mature osteoblasts and adipocytes (Fig. 2e). Similarly, a clonogenic periosteal population can be identified by pulse labelling in vivo (Extended Data Fig. 2h). PSCs also possessed the capacity to differentiate into chondrocytes (Fig. 2f). In summary, PSCs are the most stem-like of the CTSK–mGFP populations in vitro.

In contrast to other skeletal mesenchymal stem cells (MSCs) that mediate endochondral ossification, PSCs form bone in vivo via an intramembranous pathway. We transplanted PSCs and non-CTSK (Lin-) MSCs under the kidney capsule of wild-type secondary recipient mice (Fig. 2f, g). Both non-CTSK MSCs and PSCs mediated de novo generation of bone organoids (Fig. 2h). Consistent with the physiologic restriction of marrow recruitment to the endosteal compartment,
non-CTSK MSCs underwent endochondral ossification with cartilage differentiation and recruitment of haematopoietic elements, whereas PSCs mediated intramembranous bone formation without haematopoietic recruitment or cartilage formation\(^1\) (Fig. 2g–j, Extended Data Fig. 3a–h). Otherwise, non-CTSK MSCs displayed similar performance to PSCs in bone organoid, clonal multipotency and mesenesophagus assays (Extended Data Fig. 3a–h). No interconversion between non-CTSK MSCs and PSCs was observed after transplantation (Extended Data Fig. 3i–k). Therefore, bone contains discrete stem cell populations with differing functional specializations\(^1\), including a PSC population specialized for periosteal physiology.

Additionally, PSCs sit at the apex of a differentiation hierarchy in vivo and are capable of self-renewal during serial transplantation assays\(^1\). CTSK–mGFP PSCs were transplanted into the mammary fat pad of female Rosa\(^2\) mice and analysed over two successive rounds of transplantation (Fig. 2). After the first round of transplantation, PSCs both self-renewed and gave rise to the entire spectrum of CTSK–mGFP cells observed in native periosteum (Fig. 2f, top panels). Similar results were observed in a kidney capsule system (Extended Data Fig. 4a).

PSCs from secondary hosts were re-isolated and transplanted into tertiary hosts where they again displayed intact self-renewal and differentiation capacity (Fig. 2k, bottom panels). By contrast, CTSK–mGFP+ PP1 or PP2 cells did not revert to PSCs after transplantation and were unable to maintain THY1.2\(^+\), GC\(^3\) progenitor cells (Extended Data Figs. 3l–n, 4b, c). Therefore, PSCs retain both self-renewal and differentiation capacity through successive rounds of transplantation.

Figure 1 | Cathepsin K-Cre labels periosteal mesenchymal cells. a, Left, mGFP (green) signal in femur/tibia of Ctsk\(^{cre}\), Rosa26\(^{mT/mGFP}\) mice at postnatal day 10 (P10). Scale bar, 500 \(\mu\)m. Right, enlarged view of dotted white box in main panel. Arrows indicate the CTSK–mGFP signal in the periosteum. b, Endosteal CTSK–mGFP cells express the osteoclast marker TRAP (magenta). Scale bar, 20 \(\mu\)m. c, Representative FACS analysis of distribution of CTSK–mGFP cells in the endosteal digest, periosteum and total bone digests. \(*\*\* P = 1.69 \times 10^{-5}\); two-tailed Student’s \(t\)-test; data are mean ± s.d.; \(n = 4\) independent experiments. d, A subset of CTSK–mGFP (green) periosteal cells in the femur expresses type 1 collagen (d: magenta, orange arrow; bottom panel shows enlarged view of outlined area), and a subset expresses CD200 (e: magenta; yellow arrows; panels on the right show enlarged views of outlined areas). Scale bar, 25 \(\mu\)m (d, top), 200 \(\mu\)m (e, top). An enlarged view of e is presented in Extended Data Fig. 1h. f, Flow cytometry of cells from long bone digests from P7 mice to identify periosteal stem cells (PSC) and progenitor cells (PP1, PP2). Colour-coded boxes (yellow, red, green) indicate parent/daughter gates. g, Percentage of PSC, PP1 and PP2 populations over time in long bone digests. PP1: \(*\*\* P = 0.0063\) (day 15), \(*\*\* P = 0.0001\) (day 32). PP2: \(*\*\* P = 0.0022\) (day 15), \(*\*\* P = 0.0001\) (day 32). One-way ANOVA, Sidak’s multiple comparison test. Data are mean ± s.d.; \(n = 5\) (days 7 and 15), \(n = 3\) (day 32); representative of 3 independent experiments. h–j, Flow cytometry for LEPR (h), CD146 (i) and CD140a (j) versus CTSK–mGFP in long bones. k, l, There are significantly fewer CD146\(^+\) (k, \(*\*\* P = 0.0000029\)) and CD140a\(^+\) (L, \(*\* P = 0.0014\)) cells among CTSK–mGFP cells than among non-CTSK–mGFP cells. Two-tailed Student’s \(t\)-test. Data are mean ± s.d.; \(n = 3\) independent experiments. Images are representative of 5 (a, b) or 3 (d, e) independent experiments. Plots are representative of \(n = 20\) (f) or \(n = 10\) (h–j) independent experiments.
Fig. 2 | Functional characterization of periosteal stem cells. a, Bright-field images, primary (left; scale bar, 20 μm) and secondary (right; scale bar, 10 μm) mesospheres derived from CTSK-mGFP PSCs (top), PP1s (middle) and PP2s (bottom). GFP (green) inset. b, Percentage of cells able to form spheres (top) and cell number per sphere (bottom) for PSCs, PP1s and PP2s. **P = 0.0036, ***P = 0.0002, ****P = 0.0001. Dunnet’s multiple comparison test. Data are mean ± s.d., n = 3 independent experiments. c, Immunostaining for CD200 (magenta) in primary (top) and secondary (bottom) PSC-derived mesospheres. Scale bars, 100 μm. d, Sorted PSCs were cultured for 15 days and analysed by FACS. e, PSC colonies, derived from single cells, were split for differentiation into osteoblasts (alizarin red staining, top; scale bar, 50 μm), adipocytes (oil red O staining, middle; scale bar, 50 μm), and chondrocytes (H&E staining of organoids derived from non-CTSK MSCs (top) or PSCs (bottom). Scale bars, 10 μm (top), 20 μm (bottom). f-j, Schematic of serial transplantation of PSCs into mouse mammary fat pad. k, FACS plots of PSC-derived cells after the first (top panels) and second (bottom panels) round of transplantation. Lin− cells are Ter119− CD45− CD31−. Colour-coded boxes (green, magenta, orange) indicate parent/daughter gates. Images are representative of 3 (a, c, e) or 8 (f-i) independent experiments. Plots in d and k are representative of 3 independent experiments.
indicating that they are at the apex of the CTSK–mGFP differentiation hierarchy.

We further examined the specialization of PSCs for intramembranous bone formation at the calvarium, a well-known site of intramembranous bone formation. Consistent with observations that calvarial sutures contain progenitors that migrate to calvarial periosteum as they mature,13,14 cells with a PSC immunophenotype exist predominantly in the sutures (Fig. 3a, c, d, Extended Data Fig. 4d, e). By contrast, CTSK–mGFP+ P1 and PP2 cells, as well as THY1.2+CD146+SCLA+ cells, were predominantly present in the calvarial periosteum outside of the sutures (Fig. 3b–d, Extended Data Fig. 4f–j). Calvarial PSCs are functionally equivalent to long bone PSCs, being at the apex of their differentiation hierarchy during in vitro and in vivo assays, and possessing similar gene expression, the capacity to form tertiary mesenchymes and bone organoids, and clonal multipotency (Extended Data Fig. 5). The few non-CTSK MSCs in the calvarium displayed low in vitro bone formation capacity (Extended Data Fig. 5d–f). Therefore, PSCs are present in the calvarial sutures, suggesting that PSCs orchestrate intramembranous ossification at multiple anatomic sites.

Fig. 3 | Periosteal stem cells in mouse calvarium and gene expression analysis of mouse femoral periosteal cells. a, CTSK–mGFP cells (green) in mouse calvarium at P15 (top) and P32 (bottom). White arrow, sagittal suture; blue arrow, calvarial periosteum; scale bar, 200 µm. Images are representative of 3 independent experiments. b, THY1.2 expression in CTSK–mGFP cells from P6 suture (left) and calvarial periosteum (right). c, PSC, P1 and PP2 in suture (left) and calvarial periosteum (right) at P6. Representative plots from 10 independent experiments. d, Relative proportion of PSC, P1 and PP2 cell populations at day 6 in suture and calvarial periosteum. *P = 0.04, **P = 0.002, ***P = 2.7 × 10−7. Two-tailed Student’s t-test. Data are mean ± s.d.; n = 3 independent experiments, 5 animals pooled per group. e, Principal component analysis of RNA-seq following FACS of PSC, P1, PP2 and non-CTSK MSC populations from P6 mouse femurs. n = 4 mice per group. f, Heat map of gene expression in PSCs and non-CTSK MSCs. g–k, CTSK–mGFP+ mesenchymal cells (n = 658) isolated by FACS from P6 mouse femur and analysed by CEL-Seq2. g, t-distributed stochastic neighbour embedding (t-SNE) of global gene expression. Cluster 1, 276 cells; cluster 2, 215 cells; cluster 3, 145 cells; cluster 4, 16 cells. h, Relative expression of Col2a1, Sox9, Kera, Alpl, Ly6a and Acta2 among the four clusters in g. i, j, Monocle analysis of the CEL-Seq2 data. i, Single-cell trajectory obtained via unsupervised ordering of 658 CTSK–mGFP cells based on state (top) and pseudotime (bottom). j, Labelling on the differentiation trajectory of PSCs (top), THY1.2+ (middle) and SCA1+ (bottom) cells, determined by FACS analysis. k, Monocle analysis for relative gene expression versus pseudotime. l, Heat map of differentially expressed genes across pseudotime.
Fig. 4 | PSCs contribute to bone formation and fracture healing; human samples contain PSC-like cells. a, b, Micro-computed tomography (µCT) of skull (a) and femur (b; smaller panels on right, enlarged view of outlined regions; arrows in iv indicate pitting that occurs on the surface of Osx\(^{+/+}\);Ctsk\^{cre}\) mice of 4-week-old mice. Scale bars, 1 mm. c, µCT of femur cortex. Red arrows indicate cortical porosity. d, Left, haematoxylin and eosin staining of mouse periosteum; right, enlarged view of outlined region. Scale bar, 20 µm. n = 5 animals per group. e, Calcein labelling of mouse femur (left) and bone formation rate/bone surface (BFR/BS; right) at 4 weeks old. Scale bar, 10 µm. *P = 0.021, **P = 0.007; two-tailed Student’s t-test. Data are mean ± s.e.m., n = 5 animals per group, 5 independent experiments. f, g, FACS analysis of cells isolated from contralateral (top) and fractured femurs (bottom) (f, green outlines indicate parent/daughter gates) and cell counts (g). *P = 0.018; **P = 0.009 (callus tissue 8 days after fracture). Two-tailed Student’s t-test. Data are mean ± s.e.m., n = 5. h, Ctsk\^{cre}\;Rosa26\text{-}mT/mG mouse femurs 9 days after fracture (top). Bottom panels show enlarged views of outlined regions. Scale bar, 500 µm. i, Alizarin red staining of PSCs from fractured (bottom) or contralateral (top) femur. Scale bar, 200 µm. j, Safranin O (left) and von Kossa staining 4 weeks after transplantation of PSCs from fractured (bottom) or contralateral femurs (top). k-n, Femur 3 weeks after fracture. k, µCT. n = 8 mice per group. Scale bar, 1 mm. l, Haematoxylin and eosin staining of fracture callus. Scale, 200 µm. m, Fracture non-union. *P = 0.025; Fisher’s exact test. n = 7 (Osx\^{+/+};Ctsk\^{cre} and Osx\^{+/+};Ctsk\^{cre}), n = 8 (Osx\^{+/+}; Ctsk\^{cre}). n, Callus bone volume. *P = 0.042. Two-tailed Student’s t-test. Data are mean ± s.e.m. n = 4 (Osx\^{+/+};Ctsk\^{cre} and Osx\^{+/+};Ctsk\^{cre}), n = 5 (Osx\^{+/+};Ctsk\^{cre}). o–r, Human femoral periosteum. o, Haematoxylin and eosin staining (left; scale bar, 200 µm), and immunohistochemistry for cathepsin K (middle, red arrows) and CD200 (right). Middle and right panels show enlarged view of the region outlined in green in the left panel. p, FACS analysis. Representative plot from n = 10 experiments. q, Alizarin red (left, scale bar, 200 µm; inset shows lower magnification), oil red O (white arrows; middle; scale bar, 50 µm) and alcian blue (right; scale bar, 200 µm) staining of cultured h-PSCs. r, Von Kossa staining (black) of bone organoids in xenografts of h-PSC (left), human P1 (h-P1) (middle) and human PP2 (h-PP2) (right). Scale bars, 20 µm. Representative images from 3 (h–j, l, r, q) or 4 (o) independent samples.
Transcriptional analysis shows that PSCs from mouse femur are broadly different from both their PP1 and PP2 derivatives and other skeletal MSCs (Fig. 3e, Extended Data Fig. 6a, g). Both PSCs and non-CTSK–mGFP MSCs share expression of genes associated with mesenchymal stem or progenitor cells, including Runx2 and Sox9, alongside stemness-associated genes such as Myc15 and Klf416, whereas PSCs expressed higher levels of Nanog and Wnt5a17 (Fig. 3f). Notably, PSCs displayed increased per-cell bone formation capacity compared to PP1/PP2 cells after kidney capsule transplantation (Extended Data Fig. 6b–d). Calvarial PSCs also displayed similar patterns of gene expression as those observed in femoral PSCs (Extended Data Fig. 5g). In parallel, to empirically determine the populations present within the pool of femoral CTSK–mGFP+ Lin− cells, we performed single-cell RNA sequencing (RNA-seq) analysis using CEL-Seq218. Mesenchymal CTSK–mGFP+ cells clustered into four groups: a group expressing progenitor/stem cell markers19 such as Sox9 and Col2a1, a group expressing osteoblast markers such as Bglap and Alpl, a group expressing Lyp6a (also known as Scal) and a small group with high expression of Acta2 (Fig. 3g, h, Extended Data Fig. 6e, f). Unsupervised construction of an inferred differentiation trajectory using Monocle20 empirically identified a population corresponding to the PSCs obtained by FACs (Fig. 3i–j). Transcriptional markers of mesenchymal stem or progenitor cells were expressed in the early part of this differentiation trajectory containing PSCs (Fig. 3k, l). Therefore, combined index sorting and single-cell RNA-seq independently identify a discrete PSC population similar to that identified by FACs, and PSCs display transcriptional signatures of mesenchymal stem cells and are distinct from other CTSK–mGFP cell populations.

To evaluate the physiologic importance of PSC-derived osteoblasts to bone formation, we blocked the ability of PSCs to give rise to osteoblasts by conditionally deleting the osterix gene (Oxs; also known as Sp7), which encodes a transcription factor essential for osteoblast differentiation21–23, using CtskCre. Oxs−/−CtskCre+ mice displayed hypomineralization of the calvarium, uneven periosteal surfaces, and extensive linear intra-cortical pores that gave a characteristic appearance of a double cortex (Fig. 4a–d, Extended Data Fig. 7a). Endosteal trabecular bone mass was not substantially altered (Extended Data Fig. 7d). Otherwise, bone morphology and growth plate architecture were intact in Oxs−/−CtskCre+ mice aside from a slight reduction in long bone length (Extended Data Fig. 7b, c). Bone formation rates were significantly reduced in the periosteum of Oxs−/−CtskCre+ mice, with a compensatory increase in endosteal bone formation (Fig. 4e, Extended Data Fig. 7e). TRAP staining showed no changes in osteoclast numbers24 (Extended Data Fig. 7f, g). Consistent with histomorphometry results, the in vivo and in vitro osteogenic potential of both long bone and calvarial PSCs but not endosteal MSCs was reduced in Oxs−/−CtskCre+ mice (Extended Data Fig. 7h–j). Therefore, PSC-derived osteoblasts are necessary for peristomial bone formation and the establishment of normal cortical architecture.

Consistent with observations that the peristiole is necessary for fracture repair24,25, PSC-derived osteoblasts expand in response to injury and are necessary for fracture healing. PSCs exhibited a greater expansion than non-CTSK MSCs in femurs 8 days after fracture (Fig. 4f, g, Extended Data Figs. 7k–p, 8, 9a–c). PSCs isolated from the fracture site displayed enhanced osteoblast differentiation capacity on a per-cell basis in vitro (Fig. 4i). Therefore, fracture markedly increases the number and the osteoblast differentiation capacity of PSCs.

It is a longstanding apparent contradiction that peristiole undergoes intramembranous bone formation at baseline while also being necessary for endochondral fracture repair. We examined whether PSCs could explain this apparent contradiction by switching from a baseline intramembranous bone formation capacity to acquire endochondral bone formation capacity after fracture. No CTSK–mGFP labelling of chondrocytes is observed at baseline; however, CTSK–mGFP cells contributed approximately half of the chondrocytes present in the fracture callus (Extended Data Figs. 8a, 9d, e). PSCs isolated from the fracture callus mediated endochondral ossification after transplantation into the kidney capsule (Fig. 4h, j, Extended Data Fig. 9f, g). Therefore, whereas there is a clear distinction between intramembranous-competent PSCs and endochondral-competent MSCs at baseline, injury can introduce plasticity or interconversion between these cell types. This offers an explanation for how the peristiole can contribute to the endochondral process of fracture repair despite being specialized for intramembranous formation at baseline.

Next, we investigated the functional contribution of PSC-derived osteoblasts to fracture healing in Osxfl/fl;CtskCre+ mice, which displayed markedly impaired fracture healing with increased rates of fracture non-union and a decrease in the total fracture callus bone volume (Fig. 4k–n). Histologic analysis of the fracture callus was consistent with defects in mineralization, showing decreased bone and increased cartilage in the callus (Extended Data Fig. 10a–g).

To establish whether a population analogous to PSCs exists in humans, human periosteal tissue from femur was sectioned and stained, showing that cells express cathepsin K and CD200 (Fig. 4o). Similarly, flow cytometry revealed the presence of a population (Lin− CD90 + CD200 + CD105−) in human periosteeum bearing an immunophenotype similar to that of mouse PSCs (Fig. 4p, Extended Data Fig. 10h–k). These human PSCs were multipotent as they underwent osteogenic, adipogenic and chondrogenic differentiation in vitro (Fig. 4q). When transplanted into the kidney capsule of immunocompromised mice, h-PSCs, h-PP1 and h-PP2 cells all mediated intramembranous bone formation (Fig. 4r, Extended Data Fig. 10l). Therefore, human peristiole contains a population analogous to PSCs.

In this study, we identified a stem cell that serves as a physiologic precursor of peristomial osteoblasts, and has a critical role in both fracture healing and modelling of the bone cortex. As the bone cortex is essential for the biomechanical resistance of bone to fracture26–29, isolation of PSCs is likely to facilitate both the development of drugs that increase cortical bone thickness as well as cellular therapy for skeletal injury30. Moreover, the existence of a discrete peristomial stem cell demonstrates that bone consists of multiple distinct pools of stem cell progenitors, which in turn enables the functional specialization of each of these stem cell types and their derivatives. In this regard, PSCs are specialized for intramembranous bone formation at baseline, providing a cellular basis for long-observed differences between intramembranous and endochondral bone formation. Lastly, the interconversion between PSCs and endochondral-competent populations suggests that such interconversions may occur more broadly and contribute to the cellular basis of skeletal pathology.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41586-018-0554-8.

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Author contributions S.D. initiated the study and M.B.G supervised the project. S.D. and M.B.G. conceived the project, S.D. designed, conducted experiments and analysed data. A.R.Y. performed all mouse surgeries. J.M. supervised flow cytometry, S.L., T.Z. and D.A.L. performed data analysis on bulk RNA-seq and single-cell RNA-seq, R.X., M.E. and J.-H.S. performed cell culture, RT–PCR, immunostaining and μCT analysis. N.L., Y.L. and Y.S.Y. performed μCT, histology and cryosectioning of samples. M.H., M.P.B. and J.H.H. provided access to human samples, helped with sample processing and supervised human studies. S.D. and M.B.G. prepared the manuscript. All authors read and approved the manuscript.

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METHODS

Animals. Cathespin K-Cre (Ctsc<sup>cre</sup>) mice were a gift from S. Kato (University of Tokyo). Floxed osteir (Oxy<sup>fl</sup>) mice were a gift from B. de Crombrugge (University of Texas M. S. Anderson Cancer Center). R26R<sup>cre</sup> offspring (Stock 017492), Actr<sup>cre</sup>ER<sup>α</sup> (Stock 004682), NOD/SCID/IIL2R<sup>(−/−)</sup> (NSG, Stock 005577) and Rosa26<sup>CreERT2</sup> (Stock 007676) were purchased from Jackson Laboratories. All mice were maintained on a C57BL/6J background throughout the study. All animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All animals and handled according to protocols approved by the Weill Cornell Medical College subcommittee on animal care (IACUC). For all cellular transplantation experiments, the recipient animals for each specific cell population transferred were randomized after selecting a group of gender-appropriate mice. Generally, sample sizes were calculated on the assumption that a 30% difference in the parameters measured would be considered biologically significant with an estimate of sigma of 10–20% of the expected mean. Alpha and Beta were set to the standard values of 0.05 and 0.8, respectively.

Blinding. μCT analysis, mesenhang cell analysis, histomorphometry, immuno-histochernistry were performed by individuals that were blinded to the identity of the groups.

Cell line. Validation for single-cell sorting was initially done with RAW264.7 cells from ATCC (ATCC TIB-71), a commercially available mouse macrophage cell line. These cells were not independently authenticated since they were obtained directly from ATCC and were only used to validate single-cell-sorting capabilities before experimentation. Cells were tested for mycoplasma using the PlasmoTest Mycoplasma Detection Kit from InvivoGen (rep-nt2) and PCR analysis. All cells were <0.1% contamination and free of mycoplasma. Tamoxifen injection. Actr<sup>cre</sup>ER<sup>α</sup> activity was induced at postnatal day 3. Tamoxifen (100 mg/kg) (Sigma) was prepared in corn oil and injected intraperitoneally into nursing females for 3 consecutive days. Nursing females in control groups were simultaneously injected with corn oil only. The resulting litters were killed 14 days after induction and femurs were fixed for 4 h with 4% paraformaldehyde (PFA) and processed for imaging as described below.

Human samples. This project was approved by the IRB committee of Memorial Sloan Kettering Cancer Center (MSKCC) (IRB No. 97–094). HIPAA authorization was included in the IRB protocol and all ethical guidelines conform to the 2008 Helsinki declaration. Patient consent was obtained and all identifying patient information was scrubbed at the time of sample procurement.

Isolation of mesenchymal cells. Skeletal tissue from postnatal day 7, 15 or 32 mice was subjected to both mechanical and enzymatic digestion. The harvested tissue was minced using razor blades and digested for up to 4 h with Collagenase P (1 mg/ml; Roche, cat. 11213857001) and Dispase II (2 mg/ml; Roche, cat. 04942078001) digestion buffer at 37 °C with agitation. Medium containing 2% serum was added to the digest and the tubes were centrifuged to pellet cells. The supernatant was discarded and the pellet was resuspended in 1× insulin-transferrin-selenium solution and briefly incubated for 5 min at 37 °C. Medium was added to the tube and the digested tissue was resuspended thoroughly by pipetting and then filtered through 70-µm nylon mesh. Tubes were centrifuged and the resulting cell pellet was subjected to FACS. Microdissociation of mouse periosteum, calvarial suture and calvarial periosteum was performed under a dissecting microscope. Each of the selected colonies was extracted using a cloning cylinder. The extracted cells were regrown for 3–4 days in 12-well plates and then allowed to differentiate under both osteogenic and adipogenic conditions (Gibco, A1105-01). Sorted human cells were cultured under conditions similar to those described above using a commercial medium preparation (basal medium, 05401, Stem Cell Technologies) with stimulatory supplements (05402, Stem Cell Technologies). Isolated populations was conducted under hypoxic conditions (Figs. 2d, Extended Data Figs. 2i, 3h, 5k–m). Clonal multipotency of sorted cell populations was evaluated under non-hypoxic conditions (Figs. 2e, 4q, Extended Data Figs. 3d, 5c). Mesenhang cell formation capacity was evaluated under non-hypoxic conditions (Fig. 2a, Extended Data Figs. 3f, 5a).

Mouse primary cultures were grown in complete mesencult medium (basal medium, 05501, Stem Cell Technologies) with stimulatory supplements (05502, 05500, Stem Cell Technologies). After initial cell plating, cells were left undisturbed and allowed to grow at 37 °C under humidified conditions for a week. Half of the medium was replaced every 7 days. Cells were passaged once they were 60–70% confluence. For media containing Pro-05402, Stem Pro Angiogenesis solution (Gibco, A1105-01). Sorted human cells were cultured under conditions similar to those described above using a commercial medium preparation (basal medium, 05401, Stem Cell Technologies) with stimulatory supplements (05402, Stem Cell Technologies).

Mesenhang cell assays. For mesenhang cell assays, FACS-isolated single cells were plated at a density of 100 cells per cm<sup>2</sup> and allowed to grow in ultra-low adherence culture dishes (Stem Cell Technologies, 27145). Plates were incubated at 37 °C with 5% CO<sub>2</sub> and left undisturbed for a week. Half of the medium was replaced every 7 days. Mesenhang cells were dissociated into single cells using Accutase solution (Gibco, A1105-01) and were subsequently re-plated to generate secondary and tertiary mesenhangs.

Clonal differentiation assay. The differentiation potential of 10 colonies derived from single FACS-isolated PSCs was examined (Fig. 2e). In brief, single FACS-sorted cells were plated at a density of 100 cells per 10-cm dish and allowed to form individual colonies. Initial dispersion of the plated cells as single cells was confirmed by light microscopy. Each of the selected colonies was extracted using a cloning cylinder. The extracted cells were regrown for 3–4 days in 12-well plates and then allowed to differentiate under both osteogenic and adipogenic conditions (Gibco, A1105-01) and were subsequently re-plated to generate secondary and tertiary mesenhangs.

Chondrogenic differentiation and alcian blue staining. Micromass cultures were generated by seeding 1 × 10<sup>4</sup> cells in 5–10 µl of basal medium. Cells were allowed to adhere to a glass slide for 2 h under highly humidified conditions at 37 °C. Chondrogenic differentiation medium (Stem Pro Chondrogenesis Differentiation kit, A10072-01) was slowly added and cells were incubated at 37 °C with 5% CO<sub>2</sub>. Cultures were re-fed every 2–3 days and allowed to differentiate for a minimum of 14 days. At the end point of this study, medium was removed, and cells were washed with PBS and fixed with 4% PFA for 30 min at room temperature. Cells were rinsed again with PBS and stained for 30 min with oil red O working solution (3:2 dilution with water). Cells were then observed under a light microscope after 4–5 washes with PBS.

Chondrogenic differentiation and alcian blue staining. Micromass cultures were generated by seeding 1 × 10<sup>4</sup> cells in 5–10 µl of basal medium. Cells were allowed to adhere to a glass slide for 2 h under highly humidified conditions at 37 °C. Chondrogenic differentiation medium (Stem Pro Chondrogenesis Differentiation kit, A10072-01) was slowly added and cells were incubated at 37 °C with 5% CO<sub>2</sub>. Cultures were re-fed every 2–3 days and allowed to differentiate for a minimum of 14 days. At the end point of this study, medium was removed, and cultures were washed with PBS and fixed with 4% PFA for 30 min. Cultures were stained for 30 min with 1% alcian blue solution, washed three times with 0.1 N HCl and then with PBS.

Clonal differentiation assay. Sorted cells were cultured both in hypoxic (2% O<sub>2</sub>, 10% CO<sub>2</sub>, 88% N<sub>2</sub>) and non-hypoxic conditions (20% O<sub>2</sub>). In vitro differentiation of FACS-isolated populations was conducted under hypoxic conditions (Fig. 2d, Extended Data Figs. 2i, 3h, 5k–m).

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solution. Sections, 10 µm in thickness, were cut and acian blue staining was performed as described.

**Surgical procedures.** All surgical procedures were performed under isoflurane (1–4%) anaesthesia. Surgical sites were sterilized using a betadine/iodide/isopropanol prep after hair removal using a clipper with a no. 40 blade and a depilatory cream (Nair). After surgery, the visceral lining or muscle was sutured with absorbable Ethicon vicryl sutures (VWR, 93057-014) before closing the skin with wound clips that were removed 2 weeks post-operatively. Animals received intraperitoneal buprenex (0.5 mg/kg) and oral meloxicam (2 mg/kg) as analgesia before surgery and for every 24 h post-surgery for 3 days. All surgical procedures are approved by the institutional animal care and use committee at Weill Cornell Medical College.

**Kidney capsule transplantation model.** In brief, 8–10-week-old male mice were anaesthetized and shaved on the left flank and abdomen before sterilization of the surgical site. The kidney was externalized through a 1-cm incision and a 2-µm pocket was made in the renal capsule. A 5–µl Matrigel plug (Corning, 356231) containing 8,000–10,000 cells was implanted underneath the capsule and the hole was sealed using a cauterizer before replacing the kidney back into the body cavity. Recipients for these experiments were syngenic with donors. Moreover, to avoid potential immunogenicity of GFP itself, all transplantation studies of CTSK–mGFP+ cells were conducted in mTmG hosts that have baseline immunologic tolerance to GFP variants. Animals were euthanized by CO2 after 6 weeks. After death, kidneys were fixed with 4% PFA for 5 h and bone formation was detected by µCT. Samples were subjected to infiltration, embedding and sectioning as described above. Haematoxylin and eosin and Von Kossa staining were performed following previously described protocols. Standard safranin O stain and alizarin red staining was performed to detect cartilage and bone components.

Altogether, over 30 individual mouse transplantation procedures were conducted across 5 separate days of experimentation to study the intramembranous versus endochondral differentiation preferences of PSCs versus non-CTSK MSCs.

**Mammary fat pad transplantation model.** To facilitate distinguishing host cells from CTSK–mGFP+ transplanted cells, 3–4-week-old female mTmG mice were used, where all host cells were mTomato+. These mice were anaesthetized and their lower abdomen was shaved before sterilization of the surgical site. A 1–1.5-cm longitudinal incision was made along the midline of the body wall below the sternum and just above the pelvic region. A second 0.5-cm cut was made from the bottom of the midline incision towards the hip of the right hind limb. The skin was carefully pulled away from the peritoneum to expose the inguinal mammary gland. A 2-µm pocket was created in the mammary fat pad just below the lymph node and a 5–µl Matrigel plug with the desired number of cells was implanted into the pocket. The pocket was sealed by clamping the edges of the cut fat pad together with a toothed forcep. Wound clips were used to close the skin incisions.

Animals were euthanized by CO2 at 2.5 weeks post-surgery, and the mammary fat pad was extracted, minced with razor blades and digested at 37°C in digestion buffer containing 1 (mg/ml) and Dispase II (2 mg/ml) in 2% serum medium for 30–45 min. Medium was added and the tubes were spun for 10 min at 1,500 r.p.m. The supernatant was carefully removed and DNAse I solution was added for 5 min without disturbing the pellet. The reaction was terminated by diluting the digestion solution with medium. Tubes were again spun for 10 min and the resulting pellet was prepared for FACs analysis.

**Femur fracture model.** In brief, after anaesthesia, an incision was made above the right anterolateral femur after sterilization of the surgical site. The femur and patella were then exposed and a 27-gauge syringe needle was inserted parallel with the long axis of the femur through the patellar groove into the marrow cavity. The needle was then removed and a single cut in the mid-diaphysis of the femur was made using a Dremel saw with a diamond thin cutting wheel (VWR, 100230-724). A blunt 25-gauge needle was then reinserted into the marrow space through the hole made in the femur to stabilize the fracture. This needle was then trimmed so it would not project into stifle joint space. Muscle was then placed over the injury site and stitched with absorbable sutures before closing the skin with wound clips. We performed femur fracture in 6-week-old Csk−/−mTmG mice (Fig. 4f–h) and 4-week-old Ovx+/+ Csk−/− mice (Fig. 4k). Mice were killed by CO2 asphyxiation after 3 weeks.

**Sample preparation for cryo-sectioning.** Freshly extracted mouse samples were fixed with 4% PFA for 4 h at 4°C. Samples were washed with PBS and decalcified with 0.5 M EDTA for 1–5 days depending on the age of the sample. Samples were incubated with infiltration solution (20% sucrose + 2% polyvinylpyrrolidone in PBS) with rocking until they sank to the bottom of the tube. Embedding was performed with OCT + 15% sucrose and samples were preserved at −80°C. Sections, 10–20 µm in thickness, were cut using a Leica cryostat.

**Immunohistochemistry.** Frozen samples were thawed at room temperature and rehydrated with PBS + 1% Triton X-100 for 15 min and blocked for 1 h at 5% donkey serum in PBS (blocking buffer). Dilutions of primary antibodies were freshly prepared in blocking samples. Samples were incubated overnight with primary antibodies at 4°C, then washed three times with PBS. Secondary antibodies (1:2,000 dilution) were added to the sample for 1–2 h, followed by washing three times with PBS. DAPI (300 nM) was added for 5–10 min and the samples were mounted with antifade mounting solution (Life technologies, P36907). Imaging was performed with a Zeiss LSM 880 with Airyscan high-resolution detector confocal microscope. For immunohistochemistry of mesospheres, mesospheres were extracted and allowed to adhere on glass chamber slides for 4 h. Spheres were fixed with 4% PFA for 15 min at room temperature, washed twice with PBS + 0.5% Triton X-100 for 15 min and blocked for 1–2 h with blocking buffer (PBS + 2% BSA + 10% horse serum + 0.5% Triton X-100). Primary antibody dilutions were prepared in blocking buffer, and added to the spheres followed by overnight incubation at 4°C. Samples were washed three times with PBS and incubated with secondary antibody for 1 h and DAPI solution for 10 min. Samples were subsequently processed for imaging as described above.

**Primary antibodies for immunohistochemistry.** Primary antibodies used were specific for collagen type I (Abcam, ab34370, 1:100 dilution), collagen type II (Millipore, ABAB887, 1:100 dilution), murine CD200 (Abcam, ab33734, 1:100 dilution), nestin (Abcam, ab11306, 1:200 dilution), gremlin 1 (Abcam, ab199267, 1:50 dilution), COMP (Abcam, ab7452, 1:50 dilution), Aggrecan (Abcam, ab3778, 1:100 dilution), THY1.2 (Invitrogen, 14-0092-82, 1:50 dilution), 6C3 (Invitrogen, 14-5891-82, 1:50 dilution), CD105 (Abcam, ab107595, 1:100 dilution), Runx2 (Abcam, ab76956, 1:200 dilution), Alpl (Abcam, ab108337, 1:100 dilution), osteocalcin (Abcam, ab93876, 1:100 dilution), CD146 (Abcam, ab75769, 1:100 dilution), CD140a (Abcam, ab96569, 1:100 dilution), CD200 (Abcam, ab203887, 1:200 dilution), trarate resistant acid phosphatase (TRAP) (Abcam, ab185716, 1:50 dilution), and cathepsin K (Abcam, ab19027, 1:200 dilution).

**Calcein labelling.** Calcein stock solution (2.5 mg/ml) was freshly prepared in PBS + 2% NaHCO3 + 150 mM NaCl). Four-week-old Ovx−/−flkTCsKm mice from the same litter were given subcutaneous calcein injection (10 mg/kg) followed by a second injection after 2 days. Mice were euthanized after 24 h, fixed overnight in 10% formalin solution and then stored in 70% ethanol. Non-decalcified femur sections were prepared for plastic embedding according to a previously published protocol.

**Histomorphometry.** Mice underwent dual calcein labelling, and uncalledcalculated femur sections were prepared for analysis and stained for TRAP as previously described. Dynamic histomorphometry parameters were measured using the OsteoMeasure Analysis system (Osteometrics) and reported following standard nomenclature. Mineral apposition rate (MAR; µm day−1), bone formation rate (bone surface (BFR/BS); µm2 day−1), bone volume (BV; mm3), bone volume/total volume (BV/TV), osteoclast number/bone perimeter (No. Oc/B. Pm) were performed using DESeq2 package. The heatmap was generated using heatmap.2 in R (ggplot2 package, where the expression values were normalized per gene over all samples. For each gene, the mean and standard deviation (s.d.) of expression over all samples was calculated, and the expression value underwent linear transformation using the formula (RPKM − mean)/s.d. For 16 bulk RNA samples, the average raw reads per sample was 46.2 million.

**Single-cell RNA-seq using CEL-Seq2.** Validation for single-cell sorting was initially done with RAW264.7 cells using 384-well plates. Single-cell sorting was confirmed through observation of growth of a single colony from the sorted cells (Extended Data Fig. 6f). We found that cells were sorted into wells at 93% efficiency and the doublets were detected in less than 2% of cases (Extended Data Fig. 6i). For the experiment, CTSK−mGFP+ cells isolated from a 6- day-old mouse femur were sorted by FACS (Becton-Dickinson Influx) and plated into individual wells of a 384-well plate pre-loaded with unique barcoded reverse-transcription primers. After sorting, cells were snap-frozen on dry ice before being submitted to the New York Genome Center (NYGC) for RNA sequencing. Single-cell RNA sequencing was performed in individual wells using a template-switching mechanism followed by PCR amplification. All barcoded amplified cDNA was then pooled and run on a pico-green assay and fragment analyzer to evaluate sample concentration.
and quality, respectively. Libraries were generated from 300 pg of pooled cDNA using the Nextera XT library preparation kit (Illumina). The library was then sequenced with custom sequencing primers across two lanes of a 25 bp × 75 bp rapid run (HiSeq 2500 instrument). Read 1 was composed of the cell barcode and unique molecular identifier (UMI), whereas read 2 was used to map the transcript to the mouse genome. After sequencing, the alignment and analysis was done using the NYGC re-tooled pipeline based on Drop-seq Core tools37 (http://mccarrollab.org/dropseq/) which provided basic quality control and expression analysis. The delivered data contained FASTQ, BAM files and an expression matrix.

Seurat analysis. Seurat v.2.0.1 and R v.3.4.1 were used for analysis of single-cell RNA-seq data38. Cells expressing at least 200 but not more than 5,500 genes were retained for analysis. Genes expressed in less than 3 cells were not taken into consideration. A total of 658 single cells were analysed for the total number of UMIs per cell, percentage of mitochondrial gene expression, percentage of ribosomal gene expression, and cell cycle. Initial clustering analysis showed an even distribution of mitochondrial gene content across clusters, so this was not considered further (data not shown). Cell cycle analysis was performed using a previously published approach39. Regression was performed to remove effects associated with cell cycle state, the number of UMIs and ribosomal gene content. Clustering was performed using the first 12 principal components and clusters were visualized using t-SNE projection.

Monocle analysis. Cell trajectory analysis was performed by using Monocle on CTSK–mGFP cells. Monocle v.2.4.0 and R v.3.4.0 were used to conduct analysis on the single-cell sequencing data. Monocle analysis40–42 was performed using negbinom.size function and the data was loaded using sparseMatrix. Quality control was performed by filtering low-quality cells including dead cells, empty wells in the plate and doublets. The mRNA threshold was defined and cells with mRNA content between 35,000 through 295,000 were analysed (Extended Data Fig. 6j). The same CEL-Seq2 dataset as used above was loaded for analysis (Extended Data Fig. 6k). Cell trajectory was obtained using genes selected by a principal-component-analysis-based method for cell ordering according to previously described methods40–42. Regression was performed to remove effects associated with cell cycle state and ribosomal gene content. A list of differentially expressed genes that change as a function of pseudotime was obtained using fullModelFormulaStr. Genes that were significant at 10% false discovery rate were selected. Heat maps to visualize pseudotime-dependent genes were generated by using a subset of the significant genes.

Statistical analyses. All data are shown as the mean ± s.d. or mean ± s.e.m. as indicated. Where applicable, we first performed the Shapiro–Wilk normality test to check normality. For comparisons between two groups, a two-tailed, unpaired Student’s t-test was used. If normality tests failed, Mann–Whitney U-tests were used. For comparisons of three or more groups, one-way ANOVA was used if normality tests passed, followed by Tukey’s multiple comparison test for all pairs of groups. If normality tests failed, Kruskal–Wallis test was performed, followed by Dunn’s multiple comparison test. GraphPad PRISM v.6.0a was used for statistical analysis. P < 0.05 was considered statistically significant.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability RNA-seq and single-cell RNA-seq data have been deposited at the Gene Expression Omnibus under accession numbers GSE106237, linked to the subseries GSE106235 and GSE106236. Microscopy images are available from the corresponding author upon reasonable request.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Analysis of CTSK–mGFP cells in mouse femur. 

**a**, CTSK–mGFP mesenchymal cells (green) were visualized in the mouse long bones at E14.5. Scale bar, 200 μm. Enlarged images of areas marked by the dotted white boxes are provided in i and ii. **b**, Immunostaining for CD200 (magenta) confirmed co-localization (shown by yellow arrows) with CTSK–mGFP cells (green) in the periosteum. A separate pool of CD200⁺ cells are detected at the future primary ossification site (marked by dotted orange line). Scale bar, 20 μm. Images in a and b are representative of 3 independent experiments. **c**, CTSK–mGFP mesenchymal cells in the long bones of mice were detected by FACS at E16.5. **d**, Visualization of CTSK–mGFP cells (green) in mouse long bones at E16.5. Scale bar, 500 μm. An enlarged view of the areas marked by dotted yellow boxes are shown in i and ii. CTSK–mGFP cells (green) were detected in the mouse periosteum (i and ii). **e**, CD200 (magenta) immunostaining confirmed co-localization with CTSK–mGFP cells (green) in the periosteum (top panels). CTSK–mGFP cells in the primary spongiosa morphologically consistent with osteoclasts stained negative for CD200 (bottom panels). Scale bar, 20 μm. Images in c–e are representative of 3 independent experiments. **f**, Visualization of CTSK–mGFP cells (green) in the periosteum (dotted white line) of mouse femur at postnatal day 6 (top) and 12 (bottom). Scale bar, 20 μm. **g**, CTSK–mGFP visualization shows rare mGFP⁺ osteocytes, an enlarged view of the dotted white box is provided (i). Scale bar, 20 μm. Image representative of 3 independent experiments. Quantification of total CTSK–mGFP-labelled periosteal cells and mGFP-labelled osteocytes in the mouse femur (ii). ***P = 6.95 × 10⁻¹⁶; two-tailed Student’s t-test. Data are mean ± s.e.m., n = 12 distinct areas of periosteum from 3 independent experiments. **h**, An enlarged view from Fig. 1e. Representative images from 3 independent experiments. **i**, FACS plots showing expression of CD49f (left) and CD51 (right) in CTSK–mGFP cells isolated from long bones of 7-day-old mice. Representative plot from 5 independent experiments. **j**, Femurs from 8-week-old Ctskcre⁺ mice were immunostained for Runx2 (magenta, top), alkaline phosphatase (ALPL) (magenta, middle) and osteocalcin (magenta, bottom). Co-localization is shown by yellow arrows. Scale bar, 20 μm. Representative images from 3 independent experiments. **k**, Femurs of 12-day-old Ctskcre⁺ mice were immunostained for gremlin 1 (magenta, top) and nestin (magenta, bottom). Dotted white line indicates periosteum. Scale bar, 20 μm. Representative images from 3 independent experiments.

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Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | FACS analysis of microdissected periosteal tissue and characterization of PSCs. a, Flow cytometry of CTSK–mGFP cells microdissected from the periosteum of P7 mouse long bones, showing the distribution of PSC, PP1 and PP2 cells. b, c, Flow cytometry showing the distribution of PSCs, PP1 and PP2 cells in mouse long bones at day 15 (b) and day 32 (c). Plots in a–c are representative of results from 10 independent experiments. d, Schematic representation of the strategy used for FACS analysis of periosteal PSC, PP1 and PP2 cell populations. e, FACS plot showing the distribution of CD146 (i) and CD140α (ii) expression in bone marrow stromal cells. f, FACS plots displaying the distribution of CD140α (i) and CD146 (ii) in mouse periosteum obtained through periosteal microdissection. Plots in e and f are representative of results from 5 independent experiments. g, Mouse bone marrow immunostained for CD146 (cyan) and CD140α (magenta). Scale bar, 100 µm. Representative images from 3 independent experiments. h, Clonogenic cells detected in the periosteum (top and middle, white arrows) and perichondrium region (bottom, white arrows) of mouse femur 2 weeks after induction of β-actin-Cre with tamoxifen. Enlarged views of outlined regions are shown. Scale bar, 50 µm. Representative images from 3 independent experiments. i, FACS plots showing in vitro differentiation for PP1 (left) and PP2 (right) cells after 15 days of culture. Representative FACS plots from 3 independent experiments. j, Alizarin red staining (red) of bone 5 weeks after transplantation of non-CTSK MSCs (left) and PSCs (right) into the kidney capsule. Representative images from 5 independent experiments. Scale bar, 50 µm. k, Relative gene expression for bone-(Runx2) and cartilage-specific genes (Col2a1, Comp, Chad) 5 weeks after transplantation of PSCs and non-CTSK MSCs. Non-CTSK MSC-derived cells display significantly higher expression of cartilage specific genes than PSCs. *P = 0.003 (Col2a1), *P = 0.002 (Comp), *P = 0.002 (Chad); two-tailed Student’s t-test. Data are mean ± s.d., n = 3. l, CTSK–mGFP+ PSCs (green) were immunostained for Runx2 (magenta, top) and osteocalcin (magenta, middle and bottom) 3, 4 or 5 weeks after transplantation into the kidney capsule. Scale bar, 20 µm. Representative images from 3 independent experiments.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Functional characterization of non-CTSK MSCs, PSCs and their derivatives. a, Total numbers of PSCs and non-CTSK MSCs in mouse femurs at postnatal day 7, day 15 and day 32. Significant decreases in number of PSCs are observed at day 15 (**P = 0.006) and day 32 (**P = 0.009) compared to day 7. Significant decreases in non-CTSK MSCs are observed at day 15 (**P = 3.8 × 10−5) and day 32 (**P = 0.0003) compared to day 7. Two-tailed Student’s t-test. Data are mean ± s.d., n = 3 independent experiments; 5 animals per group for day 7, day 15; 3 animals per group for day 32. b, μCT images of the bone formed by non-CTSK MSCs (left) and PSCs (right) 5 weeks after transplantation. Representative images from 5 independent experiments. c, Quantification of bone volume when equal numbers of non-CTSK MSCs and PSCs were transplanted into secondary hosts. Data are mean ± s.e.m., n = 3 independent experiments; two-tailed Student’s t-test. ns, not significant. d, Clonal non-CTSK MSC colonies were split for differentiation into osteoblasts (left, alizarin red staining) and adipocytes (middle, oil red O staining) (scale bar, 20 μm). Separately, chondrocyte differentiation potential was assayed (right, alcian blue staining; scale bar, 100 μm). Representative images from 4 independent experiments. e, Clonal differentiation capacity of 10 colonies isolated from PSCs and non-CTSK MSCs after subsequent culture under osteogenic (left) and adipogenic (right) differentiation conditions. All 10 colonies from each population were equally multipotent. Data are mean ± s.d., n = 3 independent experiments. f, Bright-field images of primary (left), secondary (middle) and tertiary mesenspheres (right) derived from non-CTSK MSCs. Tomato red (red) expression is shown in the insets. Scale bar, 20 μm. Representative images from 3 independent experiments. g, The percentage of PSCs and non-CTSK MSCs able to form mesenspheres. *P = 0.02, one-way ANOVA, Dunnett’s multiple comparison test. Data are mean ± s.d., n = 3 independent experiments. h, FACS analysis of in vitro differentiation of non-CTSK MSCs after 15 days of culture. Red box indicates parent/daughter gate. i, FACS plots of non-CTSK MSC-derived cells after the first round of mammary fat pad transplantation. Colour-coded boxes (red and green) indicate parent/daughter gates. FACS plots in h and i are representative of 3 independent experiments. j, FACS for CD140α (i) and CD146 (ii) in PSCs after transplantation into the mammary fat pad. k, FACS for expression of GFP (i), CD140α (ii), and CD146 (iii) in non-CTSK MSCs after mammary fat pad transplantation. l, PP1 cells were transplanted into the mammary fat pad of primary hosts for 2.5 weeks and then analysed by FACS (i–iii). Colour-coded boxes (green and magenta) indicate parent/daughter gates. m, PP2 cells were isolated by FACS and implanted into the mammary fat pad of primary recipients. PP2 derived cells in primary recipients were analysed by FACS (m, i–iv), and PP2 cells were re-isolated for transplantation into secondary recipients. PP2-derived cells in secondary recipients were analysed by FACS (n, i–iv). Colour-coded boxes (green, magenta and orange) indicate parent/daughter gates. Plots in j–n are representative of results from 3 independent experiments.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Differentiation of PSCs, PP1 and PP2 cells when transplanted into kidney capsule of secondary hosts and FACS analysis of CTSK–mGFP calvarial cells. a, CTSK–mGFP⁺ PSCs (green) were immunostained for THY1.2 (magenta, top), 6C3 (magenta, middle) and CD105 (magenta, bottom) three weeks after transplantation into the kidney capsule of primary recipients. b, CTSK–mGFP⁺ PP1 cells (green) were immunostained for 6C3 (magenta, top) and CD105 (magenta, bottom) three weeks after transplantation into the kidney capsule. c, CTSK–mGFP⁺ PP2 cells (green) were immunostained for THY1.2 (magenta, top), and CD105 (magenta, bottom) three weeks after transplantation into the kidney capsule. Scale bar, 50 µm. Images in a–c are representative of 3 independent experiments. Scale bar, 50 µm.

d, e, FACS analysis of PSCs, PP1s and PP2s at P15 in mouse calvarial suture (d) and calvarial periosteum (e). FACS for THY1.2 in CTSK–mGFP cells isolated from calvarial suture (f (i), g (i)) and calvarial periosteum (f (ii), g (ii)) at day 15 (left plots) and day 32 (right plots). Plots in d–g are representative of results from 3 independent experiments.

h, CD146 and SCA1 expression in CTSK–mGFP cells from the suture (i, ii) and periosteum (iii, iv) of P6 mouse calvarium. Representative FACS plots from 10 independent experiments. i, j, FACS for CD146 (i (i), j (i)) and SCA1 (i (ii), j (ii)) in calvarial periosteum of mice at day 15 (top plots) and day 32 (bottom plots). Representative FACS plots from 3 independent experiments.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Functional characterization of CTSK–mGFP+ calvarial suture cells. a, Bright-field images of primary (left; scale bar, 10 µm) and secondary (right) mesenspheres derived from calvarial suture PSCs (top), PP1 (middle) and PP2 (bottom) cells. GFP (green) expression shown in the insets. Representative images from 3 independent experiments. b, Quantification of the percentage of PSC, PP1 and PP2 cells able to form mesenspheres. Tertiary colony formation is significantly reduced in PSCs (**P = 0.0034). PP1 and PP2 cells show significant reduction in both secondary (**P = 0.0002 for PP1 and *P = 0.016 for PP2) and tertiary mesensphere formation (****P = 0.0001 for PP1 and ****P = 0.0001 for PP2). One-way ANOVA, Dunnett's multiple comparison test; mean ± s.d., n = 3 independent experiments. c, Clonal PSC colonies were split for differentiation into osteoblasts (alizarin red staining, left) and adipocytes (oil red O staining, middle; scale bar, 10 µm). Separately, chondrocyte differentiation potential was assayed (alcian blue staining, right; scale bar, 100 µm). Representative images from 3 independent experiments. d, The amount of bone formed by PSCs, PP1, PP2 and non-CTSK MSCs 4 weeks after transplantation into the kidney capsule of secondary hosts was determined by μCT. e, Von Kossa staining (e (i), dotted box in green) displaying bone organoid formation in the kidney capsule by PSCs (top left), non-CTSK MSCs (top right), PP1 (bottom left) and PP2 (bottom right) cells. Scale bar, 50 µm. Safranin O staining (e (ii), dotted box in magenta) indicates an absence of cartilage formation (transplant area shown by dotted yellow line) after transplant of PSCs (left) and PP2 (right) cells. Scale bar, 50 µm. f, In vitro osteogenic differentiation of PSCs (left) and non-CTSK MSCs (right) as determined by Alizarin red staining (red). Images in d–f are representative of 3 independent experiments. g, Heat map generated from quantitative real-time PCR analysis shows differences in gene expression between calvarial suture PSCs and the progenitor populations, PP1 and PP2 cells. h, PSCs were transplanted into a mammary fat pad of primary hosts for 2.5 weeks and then analysed by FACS (i–iii). Colour-coded boxes (green and magenta) indicate parent/daughter gates. i, FACS analysis of PP2 cells after transplantation into the mammary fat pad of primary hosts (i–iii). j, FACS analysis shows poor engraftment and loss of PP1 cells (as detected by GFP expression) when transplanted into the mammary fat pad of primary hosts. k–m, FACS plots demonstrating the in vitro differentiation of PSC (k), PP1 (l), and PP2 cells (m) when cultured for 2 weeks. Magenta boxes indicate parent/daughter gates for each analysed cell population. Plots in h–m represent results from 3 independent experiments.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Gene expression analysis in CTSK–mGFP cells isolated from mouse femurs. a, Bulk RNA-seq analysis of FACS-isolated PSC, PP1, PP2 and non-CTSK–mGFP MSCs from 6-day-old mouse femurs. Hierarchical clustering analysis was performed on RNA-seq data. b, Heat map generated from bulk RNA-seq of FACS-sorted cells shows differences in gene expression between PSCs and the progenitor populations, PP1 and PP2 cells. c, Von Kossa staining indicates bone organoid formation by PSCs (left), PP1 (middle) and PP2 cells (right) 5 weeks after transplantation into the kidney capsule. Scale bar, 20 µm. Representative images from 3 independent experiments with 3 mice per group. d, Significantly reduced bone formation (bone volume) in PP1 (*P = 0.04) and PP2 (*P = 0.032) cells compared to PSCs after transplantation. Two-tailed Student’s t-test. Data are mean ± s.d., n = 3 independent experiments. e, Relative expression of Tnn (i), Tnmd (ii), Ifitm5 (iii) and Bglap (iv) among the four clusters (identified by 1–4) that were generated through analysis of 658 CTSK–mGFP+ mesenchymal cells using the Seurat package. Cell clusters (1–4) along the x axis. f, Expression of genes such as Bglap (i), Alpl (ii), Ifitm5 (iii), Tnn (iv), Tnmd (v) and Kera (vi) are shown by pseudocolouring of t-SNE plots. g, Heat map generated from bulk RNA-seq shows differences in gene expression between PSCs and the progenitor populations, PP1 and PP2 cells. h–k, Monocle analysis of CEL-Seq2 data. h, Bright-field image of a colony that was generated from single-cell sorting of RAW264.7 cells by FACS. Scale bar, 20 µm. Representative image from 3 independent experiments. i, Graphs indicate the percentage of wells that received sorted cells by FACS (left) and the percentage of doublets detected in those wells (right). Data are mean ± s.d., n = 4. j, Data represent the total amount of mRNA in the two 384-well plates (plate 47 and plate 48) that were sequenced using CEL-Seq2. k, Pie chart, showing that the analysed CTSK–mGFP+ cells were mesenchymal in origin.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | μCT, histomorphometric analysis and characterization of cells isolated from Osx$^{fl/fl};Ctsk^{cre}$ mouse femur.

a, μCT images of longitudinal sections of femurs from Osx$^{fl/fl};Ctsk^{cre}$ mice or littermate controls at 4 weeks of age. b, Haematoxylin and eosin staining showing growth plate morphology in Osx$^{fl/fl};Ctsk^{cre}$ mice or littermate controls. Images in a and b are representative of 5 independent experiments. Scale bar, 100 μm.

c, Bone length (i), midshaft along long axis (ii) and midshaft along short axis (iii). Osx$^{fl/fl};Ctsk^{cre}$ mice show a significant reduction in bone length (i) compared to Osx$^{+/+};Ctsk^{cre}$ (*P = 0.039) and Osx$^{+/+};Ctsk^{+/+}$ (*P = 0.034). Two-tailed Student’s t-test. Data are mean ± s.d., n = 6 animals per group. d, Bone volume/total volume (BV/TV) for trabecular bone. Data are mean ± s.e.m., n = 4 animals per group, two-tailed Student’s t-test. e, Histomorphometric parameters. Cortical mineral apposition rate (MAR; μm day$^{-1}$).

*P = 0.031; two-tailed Student’s t-test; data are mean ± s.e.m., n = 5 animals per group at 4 weeks of age. f, TRAP staining of osteoclasts in the trabecular bone area of femurs of the indicated mice at 4 weeks of age. Scale bar, 100 μm. Representative images from 4 independent experiments.

g, Quantification of osteoclast number/bone perimeter (No. Oc/B. Pm). Data are mean ± s.e.m., n = 4 animals per group, two-tailed Student’s t-test. h, μCT images showing the amount of bone formed when periosteal PSCs (left column) and endosteal MSCs (right column) isolated from femurs of Osx$^{+/+};Ctsk^{cre}$ (top) and Osx$^{fl/fl};Ctsk^{cre}$ mice (bottom) were transplanted into the kidney capsule. Scale bar, 1 mm.

i, Von Kossa staining (black) of bone organoids formed after transplantation of periosteal PSCs (left column) and endosteal MSCs (right column) isolated from Osx$^{+/+};Ctsk^{cre}$ (top) and Osx$^{fl/fl};Ctsk^{cre}$ mice (bottom) and transplanted into the kidney capsule. Scale bar, 20 μm.

Images in h and i are representative of 3 independent experiments. j, Alizarin red staining (red) of periosteal PSCs (left column) and endosteal MSCs (right column) isolated from the femur (i, ii) and calvarial sutures (iii, iv) of Osx$^{+/+};Ctsk^{cre}$ (top panel) and Osx$^{fl/fl};Ctsk^{cre}$ mice (bottom panel) after culture under osteoblast differentiation conditions. Images are representative of 3 independent experiments. k, l, FACS plots of contralateral unfractured femurs (k) and fractured femurs (l). Colour-coded boxes (red) indicate parent/daughter gates. Representative FACS plots from 3 independent experiments. m, A significant increase (*P = 0.019) is seen in non-CTSK MSCs in callus tissue 8 days post fracture. Values displayed represent the absolute number of cells isolated per fracture callus. Data are mean ± s.d., n = 4 independent experiments, 4 animals/group; two-tailed Student’s t-test. n, Graph displays significantly (*P = 0.017) higher fold PSC count than non-CTSK MSCs in the fractured callus. Data are mean ± s.d.; n = 3 independent experiment; two-tailed Student’s t-test. Values displayed are normalized relative to the pre-fracture numbers of the same corresponding population to demonstrate the fold expansion after fracture. o, p, FACS of cells from fractured callus after 3 (o) and 6 days (p) of fracture. Colour-coded boxes (green) indicate parent/daughter gates. Representative FACS plots from 3 independent experiments.

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Extended Data Fig. 8 | Ctsk^Cre/mTomG mouse femur 6 days and 15 days after fracture. a, Mouse femur 6 days after fracture (top). Bottom, enlarged view of areas indicated by dotted white boxes. CTSK–mGFP (green), mTomato red (red). Scale bar, 500 μm. Images are representative of 3 independent experiments. b, Mouse femur 15 days after fracture (top). Bottom, enlarged view of areas indicated by dotted white boxes. Scale bar, 500 μm. Images are representative of 3 independent experiments.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | Characterization of CTSK–mGFP cells of mouse femur after fracture. a, The periosteum of mouse femur 6 (left), 9 (middle) and 15 (right) days after fracture. b, Haematoxylin and eosin staining of callus tissue 6 (top), 9 (middle) and 15 (bottom) days after fracture. c, CD200 (magenta) immunostaining of femurs collected 6 (top and middle) and 9 (bottom) days after fracture. d, Immunostaining for type 2 collagen (magenta) 9 days after fracture. e, TRAP staining (magenta), identifying osteoclasts in the bone callus (top) and bone marrow (middle) of fractured femurs. Few to no TRAP-positive cells were present in the periosteal region (bottom). Images in a–e are representative of 3 independent experiments. f, PSCs isolated from fracture callus were transplanted into kidney capsule secondary hosts. µCT images of bone formation at 3 (left), 4 (middle) and 5 weeks (right) after PSC transplantation to the kidney capsule (i). Safranin O staining (red), and Von-Kossa staining (black) were performed on sectioned kidney samples to detect cartilage and bone at 3 (ii), 4 (iii) and 5 (iv) weeks after PSC transplantation. Scale bar, 10µm. Haematoxylin and eosin staining indicates that PSCs isolated from fracture callus are competent to recruit host-derived haematopoietic elements at the site of transplantation (yellow arrows, v). g, Immunostaining reveals co-localization of CTSK–mGFP + cells (green) with cartilage-specific markers such as COMP (magenta, top and middle) and aggrecan (magenta, bottom) 4 weeks after PSC transplantation. Scale bar, 20µm. Images in f and g are representative of 3 independent experiments.
Extended Data Fig. 10 | See next page for caption.
Extended Data Fig. 10 | Characterization of Osx<sup>fl/fl;Ctsk<sup>cre</sup> mice and human periosteal cells. a, µCT images of Osx<sup>fl/fl;Ctsk<sup>cre</sup> mice 12 days post-fracture. Scale bar 1 mm. b, Safranin O staining was performed to detect cartilage in the callus 12 days after fracture. Images in a and b are representative of 3 independent experiments. c, Significantly higher amounts of cartilage were detected in Osx<sup>fl/fl;Ctsk<sup>cre</sup> mice compared to Osx<sup>fl/+;Ctsk<sup>cre</sup> (P = 0.035) and Osx<sup>+/+;Ctsk<sup>cre</sup> (P = 0.04) mice. Two-tailed Student’s t-test; data are mean ± s.d., n = 3 independent experiments, 3 mice per group. d, Haematoxylin and eosin staining of callus tissue 12 days after fracture. Representative images from 3 independent experiments. e, Significantly lower bone volume (BV) was detected in Osx<sup>fl/fl;Ctsk<sup>cre</sup> mice compared to Osx<sup>fl/+;Ctsk<sup>cre</sup> (***P = 0.0002) and Osx<sup>+/+;Ctsk<sup>cre</sup> (**P = 0.002) mice. Two-tailed Student’s t-test; data are mean ± s.d., n = 3 independent experiments, 3 animals per group. f, Safranin O staining for callus tissue 3 weeks after fracture. Representative images from 3 independent experiments. g, Significantly higher amounts of cartilage were detected in Osx<sup>fl/fl;Ctsk<sup>cre</sup> mice compared to Osx<sup>fl/+;Ctsk<sup>cre</sup> (***P = 0.0005) and Osx<sup>+/+;Ctsk<sup>cre</sup> (***P = 0.0003) mice at 3 weeks after fracture. Two-tailed Student’s t-test; data are mean ± s.d., n = 3 independent experiments; 6 mice for control, 5 mice each for heterozygote and knockout. h–i, FACS using CD49f, CD51 (h) LEPR (i) and CD146 (j) in human periosteal cells obtained from the femur. Representative FACS plots from 10 independent experiments. k, In vitro differentiation of h-PSCs (k, i, ii) and h-PP1 cells (k, iii, iv) after 3 weeks of culture. Colour-coded boxes (green) indicate parent/daughter gates for each cell type. Representative FACS plots from 3 independent experiments. l, Safranin O staining showing an absence of cartilage formation after h-PSC (left), h-PP1 (middle) and h-PP2 (right) transplantation into the kidney capsule of immunocompromised mice. The area containing the transplanted tissue is shown by the dotted yellow line. Representative images from 3 independent experiments.
Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

| n/a | Confirmed |
|-----|-----------|
| ☑   | The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement |
| ☑   | An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
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| ☑   | Give P values as exact values whenever suitable. |
| ☑   | For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
| ☑   | For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
| ☑   | Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated |
| ☑   | Clearly defined error bars |
| ☑   | State explicitly what error bars represent (e.g. SD, SE, CI) |

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

Dynamic histomorphometry parameters were measured using the Osteomeasure Analysis system (Osteometrics). FACS data was collected with FACS Aria II using BD FACS Diva Software Version 8.0.1 (build 2014 07 03 11 47) Firmware version 1.8 (BD FACS Aria II).

FACS data was collected with Influx using BD FACS Software 1.2.0.142, Client Application: BD FACS Software 1.2.0.142, Server Application: Utopex 1.2.0.108, Firmware version: 7.5.1.3.16.

Imaging was performed with Airyscan confocal microscopy using the ZEN 2.3 software.

Data analysis

GraphPad PRISM software (v6.0a, La Jolla, CA) was used for statistical analysis.

FACS data was analyzed using FlowJo (version 10.1).

Seurat version 2.0.1 and R version 3.4.1 were used for analysis of single cell RNA sequencing data.

Monocle version 2.4.0 and R version 3.4.0 was used to conduct analysis on the single cell sequencing data.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [x] Life sciences
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

- Generally, sample sizes were calculated on the assumption that a 30% difference in the parameters measured would be considered biologically significant with an estimate of sigma of 10-20% of the expected mean. Alpha and Beta were set to the standard values of .05 and 0.8, respectively.

Data exclusions

- No exclusion

Replication

- Yes, all experiments reported in the manuscript were replicated to confirm reproducibility. 1) FACS analysis on mouse samples included more than 35 independent experiments at the day 7 timepoint, each with consistent results. FACS analysis at other timepoints was replicated at least 3 times. 2) Analysis of human periosteum included 12 independent samples. 3) Mesensphere assays were reproduced across three independent experiments. 4) Transplantation data was reproduced at least three times. 4) Animal studies were reproduced across at least 4 independent cohorts.

Randomization

- For all cellular transplantation experiments, the recipient animals for each specific cell population transferred were randomized after selecting a group of gender-appropriate mice.

Blinding

- μCT analysis, mesensphere calculation, histomorphometry, immunohistochemistry were performed by individuals (Yeon-Suk Yang, Na Li, Mark Eiseman, Sarfaraz Lalani, Yifang Liu, Alisha Yallowitz) who were blinded to the nature of the mice under analysis (both what specific mouse strains or treatment groups were in the experiment and whether any individual mouse belonged to control versus experimental groups).

Reporting for specific materials, systems and methods

Materials & experimental systems

- n/a
- [x] Unique biological materials
- [ ] Antibodies
- [x] Eukaryotic cell lines
- [x] Palaeontology
- [x] Animals and other organisms
- [x] Human research participants

Methods

- n/a
- [x] ChiP-seq
- [x] Flow cytometry
- [x] MRI-based neuroimaging

Antibodies

- Antibodies used
  - Antibodies for FACS of human samples included a purified NA/LE Human (BD Pharmingen, Cat no. 564765, Lot no. 5274925, 1:100 dilution) Fc blocking antibody, CD235a (clone HiR2, eBioscience, REF 13-9987-80, Lot no. E03409-1633, 1:200 dilution).
Validation

The following validation methods were conducted: 1) isotype controls were used, 2) for IHC, secondary antibody was added blocking antibody, CD45 (clone 30-F11, BD bioscience, Cat no. 563891, Lot no. 7018619, 1:200 dilution), CD31 (MEC13.3, BD bioscience, Cat no. 563809, Lot no. 6193723, 1:200 dilution), Ter119 (clone Ter119, BD bioscience, Cat no. 563995, Lot no. 7023860, 1:200 dilution), CD90.2 (clone S3-2-1, BD bioscience, Cat no. 652527, Lot no. 6084947, 1:200 dilution), BP-1 (clone 6C3, eBioscience, REF 46-5891-82, E25155-101, 1:200 dilution), CD200 (clone MRC OX90, BD bioscience, Cat no. 565547, Lot no. 5281512, 1:200 dilution), CD146 (clone ME-9F1, BD bioscience, Cat no. 562230, Lot no. 5162688, 1:200 dilution), CD140α (clone APAS, BD bioscience, Cat no. 135910, Lot no. B195893, 1:200 dilution), CD49f (clone GoH3, BD bioscience, Cat no. 563707, Lot no. 6224653, 1:200 dilution), CD51 (clone RMV-7, BD bioscience, Cat no. 551380, Lot no. 6175693, 1:200 dilution), Ly6A/E (clone D7, BD bioscience, Cat no. 563991, Lot no. 6070661, 1:200 dilution), LeptinR (R&D system, Cat no. BAF497, Lot no. BVF0614101, 1:25 dilution), CD61 (clone 2C9, BD bioscience, Cat no. 562917, Lot no. 6084996, 1:200 dilution), Streptavidin eFluor 710 (eBioscience, REF 49-4317-82, Lot no. E10042-1631), NA.

Primary antibodies used for immunofluorescence and Immunohistochemistry were specific for Collagen type I (Abcam, ab34710, Lot no. GR292807-2, 1:100 dilution), Collagen type II (Millipore, MA8887, clone 683, Lot no. 2659270, 1:200 dilution), murine CD200 (Abcam, ab33734, clone MRC OX90, Lot no. GR242674-4, 1:100 dilution), Nestin (Abcam, ab11306, Lot no. GR49934-3, 1:200 dilution), Gremelin 1 (Abcam, ab189286, Lot no. GR179678-30, 1:50 dilution), COMP (Abcam, ab74524, Lot no. GR100553-1, 1:50 dilution), Agregcan (Abcam, ab3778, Lot no. 3181099-2, 1:100 dilution), Thyl.12 (Invitrogen, cat no. 14-0902-82, Lot no. 434658, 1:50 dilution), CD3 (Invitrogen, cat no. 14-5891-82, Lot no. 1948250, 1:50 dilution), CD105 (Abcam, ab107595, Lot no. GR250884-3, 1:100 dilution), Run2 (Abcam, ab76956, Lot no. GR205095-1, 1:200 dilution), Alpi (Abcam, ab108337, Lot no. GR79855-24, 1:100 dilution), Osteocalcin (Abcam, ab39378, Lot no. GR295110-2, 1:100 dilution), CD61 (Abcam, ab75769, Lot no. GR208953-3, 1:100 dilution), CD140α (Abcam, ab96569, Lot no. GR299818-1, 1:100 dilution), CD200 (Abcam, ab203887, Lot no. GR292216-1, 1:200 dilution), human CD200 (Abcam, ab203887, Lot no. GR292216-1, 1:200 dilution), Teratrate Resistant Acid Phosphatase (TRAP) (Abcam, ab185716, Lot no. GR 237040-5, 1:50 dilution), and Cathepsin K (Abcam, ab19027, Lot no. GR189688-18, 1:200 dilution).
Manufacture Primary antibodies validated for Immunofluorescence (IF) and Immunohistochemistry (IHC) (antibody, DOI/PMID):
Collagen type I (Abcam, ab34710, Lot no. GR292807-2, 1:100 dilution), 10.1007/s00441-013-1743-2
Collagen type II (Millipore, MAB8887, clone 683, Lot no. 2659270, 1:100 dilution), 10.1371/journal.pone.0087706
murine CD200 (Abcam, ab33734, clone MRC OX90, Lot no. GR242674-4, 1:100 dilution), A
Nestin (Abcam, ab11306, Lot no. GR49934-5, 1:200 dilution), 10.1172/JC89511
Gremlin 1 (Abcam, ab189267, Lot no. GR179678-30, 1:50 dilution), 10.1371/journal.pone.0176752
COMP (Abcam, ab74524, Lot no. GR100533-1, 1:50 dilution), 10.1089/ten.TEA.2011.0338
Aggrecan (Abcam, ab3778, Lot no. 3181099-2, 1:100 dilution), 10.1038/nmat4993
Thy1.2 (Invitrogen, cat no. 14-0902-82, Lot no. 4346589, 1:50 dilution), 10.1038/ncomms8398
6C3 (Invitrogen, cat no. 14-5891-82, Lot no. 1948240, 1:50 dilution), 10.1073/pnas.0610732104
CD105 (Abcam, ab117059, Lot no. GR250884-3, 1:100 dilution), 10.1111/jcmm.12762
RUNX2 (Abcam, ab76956, Lot no. GR3205095-1, 1:2000 dilution), 10.1038/ncomms3917
Osteocalcin (Abcam, ab298376, Lot no. GR295110-2, 1:100 dilution), 10.1038/ncomms9391
CD146 (Abcam, ab75769, Lot no. GR292116-1, 1:200 dilution), NA
human CD200 (Abcam, ab203887, Lot no. GR292116-1, 1:200 dilution), NA
Tartrate Resistant Acid Phosphatase (TRAP) (Abcam, ab185716, Lot no. GR237040-5, 1:50 dilution), NA
Cathepsin K (Abcam, ab19027, Lot no. GR189688-18, 1:200 dilution), 10.1038/srep25198

Manufacture tested Secondary antibodies validated for Immunofluorescence (IF) and Immunohistochemistry (IHC) (antibody, DOI/PMID)
goat anti-rabbit IgG Alexa fluor 633 (Life technologies, Cat no. A-21070, 1:2000 dilution), 10.1038/ncomms3917
goat anti-rat IgG Alexa flor 633 (Life technologies, Cat no.A-21094, 1:2000 dilution), 10.1016/j.stemcr.2015.02.014
goat anti-mouse IgG Alexa fluor 633 (Life technologies, Cat no. A-21052, 1:2000 dilution), 10.1038/ncomms9391

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
Validation for single cell sorting was initially done with RAW264.7 cells from ATCC (Cat # ATCC® TIB-71), a commercially available mouse macrophage cell line.

Authentication
Validation for single cell sorting was initially done with RAW264.7 cells from ATCC (Cat # ATCC® TIB-71), a commercially available mouse macrophage cell line.

Mycoplasma contamination
Cells were tested for mycoplasma using the PlasmoTest™ - Mycoplasma Detection Kit from Invivogen (Cat # rep-pt2) and PCR analysis. All cells were confirmed to be free of mycoplasma.

Commonly misidentified lines
No commonly misidentified cell lines were used.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals
Cathepsin K-cre (Ctsk-cre) mice were a gift from S. Kato (University of Tokyo, Tokyo, Japan). Floxed Osterix (Osxl/fl) mice were a gift from B. de Crombrugghe (University of Texas M.S. Anderson Cancer Center, Houston, Texas). CS7B6/J (Stock 000664), R26R-Confetti (Stock 017492), β-actin-cre ERTM (Stock 004682), NOD scid gamma (NSG, Stock 005557) and mTmG mice (Stock 007676) were purchased from Jackson Laboratories. All mice were maintained on a Cs7B6/J background throughout the study. All animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were handled according to protocols approved by the Weill Cornell Medical College subcommittee on animal care (IACUC) under protocol number 2012-0005. Animals were euthanized by CO2 for primary cell isolation and analyses or morphological studies.

Male and female Ctsk-cre; mTmG and Ctsk-cre; floxed Osterix mice between postnatal day 7 and 32 were used for isolation of mesenchymal cells. Male mTmG, CS7B6/J, and NOD scid gamma mice between 8 to 10 weeks were used for kidney capsule transplantsations. Female mTmG mice between 3 to 4 weeks were used for mammary fat pad transplantsations. Male and female Ctsk-cre; mTmG and Ctsk-cre; floxed Osterix mice between 4 and 6 weeks of age were used for femur fractures, which were analyzed daily to 21 days post fracture. Male and female Ctsk-cre; floxed Osterix mice were injected with Calcein and the skulls and femurs were analyzed between postnatal day 12 and 32. Immunohistochemistry was performed on male and female Ctsk-cre; mTmG mice at embryonic days 14.5 to 16.5 and at postnatal day 10. Male and female β-actin-cre ERTM R26R-Confetti were induced with Tamoxifen or oil at postnatal day 3 and were analyzed at postnatal day 17. No adverse effects were observed during this study.

Wild animals
This study did not involve wild animals

Field-collected samples
This study did not involve field-collected samples
Human research participants

Policy information about studies involving human research participants

Population characteristics

25 patients undergoing orthopedic procedures underwent periosteum harvest. This group had an age range of 18-35 (median 23), and were 67% male.

Recruitment

Participants were recruited on an "as available" basis by participating surgeons (Dr. John Healey). Recruitment bias is unlikely to impact this study as no patient comparisons are performed and no analysis of demographic or clinical covariates on cellular properties is performed.

Flow Cytometry

Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Skeletal tissue from postnatal day 7, 15 or 32 mice was subjected to both mechanical and enzymatic digestion. The harvested tissue was minced using razor blades and digested for up to 1 hour with Collagenase P (1mg/ml; Roche, cat 11213857001) + Dispase II (2mg/ml; Roche, cat 04942078001) digestion buffer at 37oC with agitation. Media containing 2% serum was added to the digest and the tubes were centrifuged to pellet cells. The supernatant was removed, and the pellet was resuspended in DNase I (2 units/ml) solution and briefly incubated for 5 mins at 37oC. Media was added to the tube and the digested tissue was re-suspended thoroughly by pipetting and then filtered thorough 70-micron nylon mesh. Tubes were centrifuged and the resulting cell pellet was subjected to FACS. Microdissection of mouse periosteum, calvarial suture and calvarial periosteum was performed under a dissecting microscope and the resulting tissue samples were subjected to the same digestion protocol. For human samples, tissue was treated with Pronase (1mg/ml; Roche, cat 10165921001) for 20 minutes at 37oC under agitation and spun down at 1500 rpm for 10 minutes prior to collagenase treatment as above. Cells were washed twice with ice cold FACS buffer (2% FBS + 1mM EDTA in PBS), incubated with blocking buffer (1:100 dilution; BD bioscience 553142 for mouse and 564765 for human) for 15 mins at 4oC. Primary antibody dilutions were prepared in brilliant stain buffer (BD bioscience 563794). Cells were incubated in the dark for 1 hour on ice with primary antibody solution, washed 2 to 3 times with FACS buffer, and incubated with secondary antibody solution for 20 minutes. Cells were then washed several times and re-suspended in FACS buffer with DAPI (4-6, Diamidino-2-Phenylindole; 1μg/ml; Invitrogen D1306).

Instrument

FACS was performed using a Becton-Dickinson Aria II equipped with 5 lasers (BD bioscience). For single cell RNA sequencing experiment, CTSK-mGFP positive cells isolated from 6-day old mouse femur were sorted by FACS (Bencton - Dickinson Influx, equipped with 6 lasers) and plated into individual wells of a 384 well-plate pre-loaded with unique barcoded RT-primers.

Software

Typically, 1 to 2 million events were recorded for each sample, and the data was analyzed using FlowJo (version 10.1).

Cell population abundance

Sorting was performed using a 4-way collection, and was validated to result in >95% purity of the intended population in postsort fractions.

Gating strategy

Beads (eBioscience 01-1111) were used to set initial compensation. Fluorescence minus one (FMO) controls were used for additional compensation and to assess background levels for each stain. Gates were drawn as determined by internal FMO controls to separate positive and negative populations for each cell surface marker. We selected live cells (negative for DAPI) and excluded doublets. Mesenchymal cell populations negative for CD45, CD31 and Ter119 cell surface markers were analyzed according to the approach described in Supplementary figure 2d.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.