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Isolation and functional assessment of mouse skeletal stem cell lineage

Gunsagar S Gulati^{1,2,5}, Matthew P Murphy^{1,2,5}, Owen Marecic^{1,2,5}, Michael Lopez^{1,2,5}, Rachel E Brewer^{1,2}, Lauren S Koepke^{1,2}, Anoop Manjunath^{1,2}, Ryan C Ransom^{1,2}, Ankit Salhotra^{1,2}, Irving L Weissman^{1,3,4}, Michael T Longaker^{1,2}, and Charles K F Chan^{1,2}

¹Stanford Stem Cell Biology and Regenerative Medicine Institute, Stanford University, Stanford, CA, USA.

²Hagey Laboratory for Pediatric Regenerative Medicine, Plastic and Reconstructive Surgery Division, Department of Surgery, Stanford University, Stanford, CA, USA.

³Department of Pathology, Stanford University School of Medicine, Stanford, CA, USA.

⁴Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA, USA.

⁵These authors contributed equally to this work.

Abstract

There are limited methods available to study skeletal stem, progenitor, and progeny cell activity in normal and diseased contexts. Most protocols for skeletal stem cell isolation are based on the extent to which cells adhere to plastic or whether they express a limited repertoire of surface markers. Here, we describe a flow cytometry–based approach that does not require *in vitro* selection and that uses eight surface markers to distinguish and isolate mouse skeletal stem cells (mSSCs); bone, cartilage, and stromal progenitors (mBCSPs); and five downstream differentiated subtypes, including chondroprogenitors, two types of osteoprogenitors, and two types of hematopoiesis-supportive stroma. We provide instructions for the optimal mechanical and chemical digestion of bone and bone marrow, as well as the subsequent flow-cytometry-activated cell sorting (FACS) gating schemes required to maximally yield viable skeletal-lineage cells. We also describe a methodology for renal subcapsular transplantation and *in vitro* colony-formation assays on the isolated mSSCs. The isolation of mSSCs can be completed in 9 h, with at least 1 h more required for transplantation. Experience with flow cytometry and mouse surgical procedures is recommended before attempting the protocol. Our system has wide applications and has already been used to study skeletal response to fracture, diabetes, and osteoarthritis, as well as hematopoietic stem cell-niche interactions in the bone marrow.

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Correspondence should be addressed to C.K.F.C. (chazchan@stanford.edu).

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Any Supplementary Information and Source Data files are available in the online version of the paper.

INTRODUCTION

Stem cell lineage trees provide a functional framework for studying the development, homeostasis, and regeneration of dynamic tissues^{1–2}. The study of skeletal development, fracture repair, bone remodeling, and osteodegenerative diseases (e.g., osteoarthritis, aging) has been limited by the crude characterization of cellular hierarchies in bone. For many decades, the gold standard for stem cell isolation from bone marrow has been to isolate cells that adhere to plastic and form colonies of fibroblast-like cells, described as mesenchymal stem cells (MSCs)^{3–6}. Several groups have further refined this definition of bone marrow MSCs by identifying gene and surface markers that label multipotential populations that form bone, cartilage, adipocytes, and other skeletal cell lineages^{7–11}. However, individual MSCs vary in their multipotency and self-renewal capacity, warranting methods to prospectively isolate stem cells with higher purity^{7,12,13}. Moreover, the lineage trajectory from MSCs to downstream differentiated cells has not been well characterized, and this has thus limited studies on stem cell, progenitor, and progeny response in normal and diseased contexts. Here, we provide a comprehensive protocol to enable researchers to study the skeletal stem cell hierarchy and hence advance understanding in the field. The protocol begins with a mechanical and chemical digestion of bone that facilitates flow-cytometric sorting based on the expression of multiple cell-surface markers. We also provide instructions for the *in vivo* and *in vitro* functional assessment of the isolated cells. This includes a model in which cells are transplanted under the renal capsule, a technique that results in improved skeletal cell engraftment, as compared with s.c. and fat-pad transplants.

Isolation of cells capable of multilineage differentiation

To enable the isolation of the different progenitor types, we initially used early fetal mouse bones, in which skeletal regenerative capacity is enriched¹⁴. We used a flow-cytometric strategy to identify a purified population of TER119[−]CD45[−] (non-hematopoietic) TIE2[−] (non-endothelial) ITGAV⁺THY1[−]6C3[−]CD105⁺ cells capable of multilineage ossicle formation *in vivo*, called mBCSPs¹⁵. The mBCSPs give rise to pro-chondrogenic progenitors (PCPs) marked by THY1⁺6C3[−]CD105⁺CD200⁺ expression; hematopoietic stem cell (HSC)-supportive osteoprogenitors and stroma, marked by THY1⁺6C3[−]CD105⁺CD200[−] (THY) and THY1[−]6C3⁺CD105⁺ (6C3) expression, respectively; B-cell lymphocyte-stimulating populations (BLSPs) marked by THY1⁺6C3[−]CD105[−] expression; and hepatic leukemia-factor-producing stroma marked by THY1[−]6C3⁺CD105[−] (HEC)^{13,15}. We later identified a CD200⁺CD105[−] precursor population that goes through a CD200[−]CD105[−] intermediate (pre-mBCSP) to then give rise to mBCSPs¹³. This CD200⁺CD105[−] cell was self-renewing, multipotent, and gave rise to all the other cells at the single-cell level; therefore, we termed it the mSSC¹³. Incidentally, despite their differences, the pre-mBCSPs and mSSCs are functionally indistinguishable in all the existing assays, so we collectively refer to the CD105[−] population as phenotypic mSSCs (p-mSSCs). All these cells are also found in adult mouse bones, although the frequency of each population changes with age.

Several groups have also found that cells distinguished by LepR; PDGFR α and Sca1 (PaS); Sca1 and CD24; Gremlin-1; and Nestin-GFP have self-renewal capacity and the capacity to differentiate to all skeletal lineages^{8,9,16–18}. These populations overlap at least partially with the cells that we term p-mSSCs and they should be considered when designing an experiment exploring skeletal stem cell fates. Compared with these other methods, we use a more restrictive gating scheme, namely CD45[–]TER119[–]TIE2[–]ITGAV⁺, to define skeletal cells and provide a wider panel of surface markers to subdivide these cells into stem cells, progenitors, and downstream progeny. Furthermore, unlike MSCs and other bone marrow stem cell populations that are only isolated by bone flush, our protocol includes a series of mechanical and chemical digestion steps to extract the skeletal lineage.

Advantages

The isolation of skeletal stem cells, the stem cells capable of self-renewal and multilineage differentiation into bone, cartilage, and stroma, has many advantages over existing models using MSCs. Unlike MSCs, which are isolated by artificial plastic adhesion and are undefined and heterogeneous, mSSCs are freshly isolated by flow cytometry and are purer and more representative of the endogenous cell types. Moreover, this protocol enables the isolation of mSSC progenitors and diverse differentiated subtypes in addition to the mSSCs themselves. These subtypes can be further studied for their roles in the HSC niche, cytokine production, and bone marrow maintenance^{19–26}. Therefore, our technique is much more comprehensive and versatile than its predecessors.

Furthermore, the study of stem cells is incomplete without functional assessment, which is why we have also described how to implement an *in vivo* transplantation assay and *in vitro* colony-formation assay for mSSCs. The renal subcapsular transplantation assay is particularly unique and advantageous over existing *in vitro* and transgenic mice model systems for the evaluation of skeletal stem cell activity. Although *in vitro* assays allow tremendous flexibility in the study of isolated subfractions of bone marrow cells and in genetically and chemically manipulating them in the dish, they do not capture the complexity of the bone and bone marrow environment and may introduce artifacts that are not representative of mSSC, mBCSP, and progeny activity *in vivo*. By contrast, transgenic mouse models, such as the inducible Cre-ER recombinase system, allow for the study of specific cells and their mechanisms in the bone and bone marrow, but are slow, expensive, and not conducive to high-throughput genetic and chemical manipulation. Fortunately, our ectopic bone-formation transplant is amenable to high-throughput genetic and chemical manipulation, and is representative of the endogenous bone marrow microenvironment. This is made possible by the flow-cytometry approach to isolating these cells, which allows sorting, manipulation, and then transplantation of these cells.

Applications

Given the versatility of the skeleton in controlling locomotion, supporting hematopoiesis^{8,15,19–26}, harboring local and metastatic cancers^{27–30}, and regulating hormones³¹, our technique has wide applications within and beyond the study of bone and cartilage biology. The isolation and transplantation of skeletal stem cells and their lineage allows for studies of the following:

- The mechanisms of stem cell self-renewal and multilineage differentiation in the bone,
- The role of skeletal stem cells, progenitors, and progeny in the physiological processes of growth and development, injury and repair, and aging, and
- The role of skeletal stem cells, progenitors, and progeny in bone cancers, such as osteosarcoma and chondrosarcoma; inherited osteochondral disorders, such as hereditary multiple osteochondromas; hematological disorders, such as Diamond–Blackfan anemia, myelodysplastic syndrome, and leukemia; and metastatic cancers of the bone, such as prostate and breast cancer.

We have previously demonstrated that in femoral fractures, a highly regenerative and osteogenic subpopulation of mBC-SPs with CD49f expression (*f*-BCSPs) is important for rapid callus formation and recovery³². In diabetes, reduced function and number of mBCSPs and mSSCs at least partially explains the poor fracture healing observed in diabetic mouse models³³. In fact, by isolating individual, purified populations of mSSCs and mBCSPs for transplant and molecular analysis, we were able to show that increased circulating TNF- α in diabetes suppresses autocrine signaling of Indian hedgehog (Ihh) in mSSCs, and that re-introduction of Ihh can reverse the poor fracture-healing defect³³. The downstream progeny of mSSCs and mBCSPs are also interesting, especially given their diverse cytokine profiles. The THY osteoprogenitors and 6C3 stroma are rich in hematopoiesis-supportive cytokines, including SCF and SDF, whereas the BLSP stroma seems to skew HSCs toward B-cell differentiation after coculture and transplant^{13,15}. Future studies could look at the molecular switches driving fate decisions in these cells, their anatomic location and migration in the bone, their aging, their susceptibility to tumorigenesis, and, perhaps most importantly, their human counterparts for therapeutic application.

We anticipate that researchers studying stem cell regeneration, developmental biology, and skeletal and hematopoietic biology would be most interested in this technique. We also believe that researchers currently using the undefined and heterogeneous MSC model to study the aforementioned processes will find our unique isolation procedure and defined cell identities useful for amending their existing models. Finally, we have used variations of this protocol to isolate other tissues in tough matrix, such as fibroblasts and tumors, so scientists looking for new and better procedures for cell isolation may find our insights useful for their experiments.

Limitations

Our system has a few limitations that must be recognized. For instance, the mSSC is likely not the only population of cells in the bone capable of self-renewal and multilineage differentiation, although it is sufficient to produce ectopic bone, cartilage, and a marrow-supportive cavity. Moreover, the mSSC does not give rise to all the cells of the bone and bone marrow; for example, they cannot differentiate into adipocytes, hematopoietic cells, or endothelium. The mSSC is also not likely to be a pure population of cells and may be composed of heterogeneous cell types, such as stem cells with biased lineage potential and different engraftment capacities. The mechanical and chemical dissociation steps outlined in our protocol itself introduce biases in the cells isolated from the bone, as cells sensitive to

the digestion buffer are killed and cells resistant to the digestion buffer are not dissociated. This can affect the fraction of cells and the types of cells isolated from the bone and bone marrow. However, within a particular age and gender of mice, the number of mSSCs and their transcriptional profile by microarray does not substantially differ between biological and technical replicates^{13–15}. The protocol is long and arduous, requiring 9 h for skeletal cell isolation, 1 h for each renal subcapsular transplantation, and days to weeks for *in vivo* renal subcapsular grafts to grow and *in vitro* colonies to form. The lengthy procedure and series of chemical reactions can subject the cells to changes in their transcription and functional activity that may differ from their endogenous properties. However, unless otherwise stated, we recommend that cells be kept on ice at all times and that viability stains be included during flow cytometry. Finally, our protocol has been optimized for C57B6/J mice, and, therefore, the percentage and types of populations isolated from different strains of mice may differ. However, we have isolated mSSCs from BALB/cJ, Rag2/gamma(c) knockout, NOD/SCID/gamma (NSG) knockout, and GFP and Actin-Cre × Rainbow³⁴ transgenic mice without compromise of mSSC number or function, demonstrating that the protocol can be used on cells from other strains of mice, even in the absence of further optimization.

Experimental design

Tissue dissociation—Skeletal-lineage cells are sensitive cells embedded in tough bone matrix, making their dissociation and isolation extremely challenging. We optimized the mechanical and chemical digestion protocols for these cells to maximize cell yield and viability. First and foremost, after dissection of the long bones from the mouse (Steps 1–6) (Fig. 1), all steps must be carried out on ice unless otherwise mentioned. Mechanical digestion with mortar and pestle must be done thoroughly to maximize the surface area for the next chemical digestion steps (Steps 7–10) (Figs. 1 and 2). The crux of the chemical digestion is the digestion buffer itself, which contains type II collagenase for the enzymatic breakdown of the cartilaginous matrix, DNase for prevention of cell clumping, CaCl₂ for enzyme stability and activity, HEPES buffer to maintain pH at 7.2–7.4, BSA for enzyme stability and medium osmolarity, and poloxamer (P-188)³⁵, a membrane sealant that protects cells from shear stress, all in Medium 199, a nutrient-rich base medium for primary cultures. The concentration of type II collagenase (3,000 U ml^{−1}) was optimized to maximize cell yield without compromising viability or surface epitopes, which can be cleaved at high collagenase concentrations. It is advised to confirm that the pH is within the normal range, especially when reusing older vials of digestion buffer, as these cells are especially sensitive to low pH. Minced bones must be digested in a shaker for 30 min (10 min to activate the enzyme plus 20 min to digest tissue) at least three times (Steps 11–15). We have found that for adult mouse bones, fewer digestions result in folds fewer numbers of cells and more digestions marginally increase yield (Supplementary Fig. 1). For mouse fetal bone samples, a single digestion is often sufficient to maximize yield. If necessary, yield can be increased by also dissecting the spine and pelvis (Table 1; results for individual mice from which the average results shown in this table were derived are provided in the Supplementary Data). We also remove red blood cells by ammonium–chloride–potassium (ACK) lysis buffer (Steps 16–23), and TER119⁺ and CD45⁺ hematopoietic cells by magnetic-activated cell sorting (MACS) (Steps 24–32) (Fig. 1). We compared this with depletion of red blood cells by density-gradient centrifugation and found that ACK lysis buffer is faster and achieves

better viability and cell yield. However, extra caution must be taken to remove bone chips from the cell solution to avoid clogging MACS columns and the flow cytometer. We suggest filtering cells through a 40-µm cell strainer and transferring the supernatant to a new tube after allowing bone chips to settle for 5 min (Steps 21 and 22).

Flow cytometry—The specific subsets of the skeletal lineage are distinguished by unique surface-marker combinations that can be exploited by flow cytometry for analysis and sorting (Fig. 1). We use an eight-color panel and a gating strategy to reproducibly isolate skeletal-lineage cells (Steps 33–41) (Fig. 3). We recommend that all gates be drawn based on fluorescence-minus-one (FMO) controls (Fig. 4), in which cells are stained with all but the one antibody of interest, using the antibody clones and fluorophores listed in both the table at Step 35 and the Reagents section. The assistance of a FACS expert is recommended for cytometer setup, fluorescence compensation, and cell sorting (Steps 42–50).

Transplantation—The renal capsule is an ideal location for skeletal lineage cell transplantation because its high blood and nutrient supply enables rapid engraftment and robust cellular growth. However, transplantation under the renal capsule is technically challenging and time-consuming. Therefore, we provide detailed instructions to increase the success rate of the transplants (Step 51A(i–xvii)) (Figs. 1 and 5). Grafts can take 4–6 weeks to grow to sizeable ossicles. Endogenous HSCs typically occupy ectopic grafts 32 d after transplantation. We recommend transplanting skeletal-lineage cells from congenic or fluorescently labeled strains to distinguish donor from recipient contributions. After graft formation, the skeletal lineage can be assessed by Movat pentachrome³⁶, immunohistochemistry, or re-dissociation and FACS analysis following the protocol described herein (Figs. 1 and 6). The use of NSG mice is useful for the study of human HSC and cancer engraftment into the ossicles^{7,20,21}.

In vitro colony-formation assay—The self-renewal and multilineage potential of skeletal cells, in addition to the lineal trajectory from mSSCs to differentiated progeny, can also be studied *in vitro* (Step 51B(i–xi)) (Fig. 1). Single mSSCs and mBCSPs give rise to visible clonal colonies in normal culture media within 2 weeks after FACS isolation. Colonies can be evaluated by differences in numbers, size, or morphology. Their composition can be assessed by alizarin red S stain for osteogenic potential, alcian blue stain for chondrogenic potential, immunohistochemistry, or re-dissociation and FACS analysis (Figs. 1 and 7). The *in vitro* system allows for the scalable expansion of mSSCs, mBCSPs, and their downstream progeny for coculture assays, transplants, and high-throughput screens. However, despite its many advantages, it is important to note that the *in vitro* culture system is an artificial environment for the study of skeletal-lineage cells, especially as we and others have shown the importance of the endogenous microenvironment in regulating normal and abnormal skeletal function^{13,15,33}.

MATERIALS

REAGENTS

- Mice: both the isolation procedure and transplant procedures described here have been optimized for normal inbred C57BL6 (JAX, cat. no. 000664), C57BL/6-Tg(CAG-EGFP)10sb/J (JAX, cat. no. 003291), and Rag2/gamma(c)KO (JAX, cat. no. 014593) male or female mice aged 8–12 weeks. Mice of different ages, genders, and backgrounds are also compatible with this protocol. Fluorescently labeled or congenic mice are advisable when performing renal subcapsular transplants to distinguish donor from recipient. ! **CAUTION** Experiments involving live rodents must conform to appropriate international, national, and institutional regulations. All animal experiments in this protocol were performed in accordance with the Stanford Administrative Panel on Laboratory Animal Care (APLAC) and received approval from the institutional review board (IRB).
- Type II collagenase powder (Sigma-Aldrich, cat. no. C6885)
- DNase I (100 mg; Worthington, cat. no. LS006344)
- FBS (Gibco, cat. no. 10437–077)
- Medium 199 with Earle's salts (Sigma-Aldrich, cat. no. M4530)
- Medium 199 with Hanks' salts (Sigma-Aldrich, cat. no. M7653)
- Minimum essential medium (MEMa) with no nucleosides (Thermo Fisher, cat. no. 12561056)
- PBS (Gibco, cat. no. 10010–023)
- Penicillin/streptomycin (Gibco, cat. no. 15140–122)
- Poloxamer 188 (100 mg ml⁻¹; Sigma-Aldrich, cat. no. P5556–100ML)
- Ammonium–chloride–potassium (ACK) lysis buffer (Gibco, cat. no. A10492–01)
- HEPES buffer (Corning, cat. no. 25–060-CI)
- BSA (Sigma-Aldrich, cat. no. A9647)
- Calcium chloride (Sigma-Aldrich, cat. no. C5670)
- Rat IgG, isotype control (Abcam, cat. no. ab37361)
- Rat anti-mouse CD90.1 (THY1.1), clone: HIS51, APC-eFluor780 (Thermo Fisher, cat. no. 47–0900-82)
- Rat anti-mouse CD90.2 (THY1.2), clone: 53–2.1 APC-eFluor780 (Thermo Fisher, cat. no. 47–0902-82)
- Rat anti-mouse CD105, clone: MJ7/18, biotin (Thermo Fisher, cat. no. 13–1051-85)

- Rat anti-mouse ITGAV (CD51), clone: RMV-7, PE (BD Biosciences, cat. no. 551187)
- Rat anti-mouse CD200, clone: OX-90, BV605 (BD Biosciences, cat. no. 745255)
- Purified rat anti-mouse CD200, clone: OX-90 (BioLegend, cat. no. 123802)
- Goat anti-rat IgG secondary antibody, Qdot 605 (Thermo Fisher, cat. no. Q-11601MP)
- Rat anti-mouse CD45, clone: 30-F11, PE-Cy5 (BioLegend, cat. no. 103110)
- Rat anti-mouse TER119, clone: TER119, PE-Cy5 (Thermo Fisher, cat. no. 15–5921-81)
- Rat anti-mouse TIE2 (CD202B), clone: TEK4 (Thermo Fisher, cat. no. 14–5987-81) ▲ **CRITICAL** to Alexa Fluor 680 fluorophore using an antibody-labeling kit such as the Alexa Fluor 680 Antibody Labeling Kit (Thermo Fisher, cat. no. A20188).
- Rat anti-mouse 6C3, clone: 6C3, Alexa Fluor 647 (BioLegend, cat. no. 108312)
- Streptavidin, PE-Cy7 (Thermo Fisher, cat. no. 25–4317-82)
- Propidium iodide (PI; InCuCyte, cat. no. 4717) ! **caution** PI is a potential carcinogen and must be handled with care. Protect the skin and eyes by wearing gloves, safety glasses, and a mask.
- Anti-CD45 microbeads, clone: 30F11, mouse (Miltenyi Biotec, cat. no. 130–052-301)
- Anti-TER119 microbeads, clone: TER119, mouse (Miltenyi Biotec, cat. no. 130–049-901)
- OneComp eBeads (Thermo Fisher, cat. no. 01–1111-42)
- AccuDrop beads (BD Biosciences, cat. no. 345249)
- Tribromoethanol (Avertin; Sigma-Aldrich, cat. no. T48402)
- Buprenorphine SR (ZooPharm)
- Povidone-iodine solution (Medline, cat. no. MDS093944H)

EQUIPMENT

- Filter-Stericup, 0.22-µm Express Plus (Millipore MP, cat. no. 163405SA)
- 40-µm Cell strainer (Falcon, cat. no. 352340)
- 100-µm Cell strainer (Falcon, cat. no. 352360)
- 1-ml Tubes (Eppendorf, cat. no. 110422–227)
- Cloning rings: cloning rings are prepared in-house from the mouths of Eppendorf tubes. Briefly, the cap is cut off from where it joins the lip of the mouth with scissors. The mouth is then cut from the rest of the tube with fine scissors.

Alternatively, glass cloning cylinders can be purchased from Sigma-Aldrich (cat. no. C1059–1EA).

- High-vacuum grease (Dow Corning, cat. no. 1597418)
- 5-ml Test tubes with cell-strainer caps (Falcon, cat. no. 352235)
- 15-ml Conical centrifuge tubes (Falcon, cat. no. 352098)
- 50-ml Conical centrifuge tubes (Falcon, cat. no. 352098)
- Size 15 scalpels (Miltex, cat. no. 4–515)
- 6–0 Black nylon suture (Ethilon, cat. no. 697H)
- Dumont 55 forceps (FST, cat. no. 11255–20)
- Fine scissors (FST, cat. no. 14958–09)
- Halsey micro needle holder (FST, cat. no. 12500–12)
- 9-mm Reflex wound clips (FST, cat. no. 12032–09)
- Wiretrol II, 10- μ l size (Drummond Scientific, cat. no. 5–000-2010)
- BioGel PI surgical gloves (Molnlycke Health Care, cat. no. ALA42675Z)
- 100-mm $\times$ 20-mm Cell culture dish, sterile (Corning, cat. no. 353003)
- Gelatin, Type B powder (Sigma-Aldrich, cat. no. G9391)
- Quadro MACS kit (Miltenyi Biotec, cat. no. 130–090-976)
- MACS multistand (Miltenyi Biotec, cat. no. 130–042-303)
- LD columns (Miltenyi Biotec, cat. no. 130–042-901)
- Certomat BS-1 incubation shaker (Sartorius, cat. no. 14–559-203)
- Floor-model centrifuge with rotor (Beckman, model no. GS-6KR)
- Flow cytometer (BD, model no. FACS Aria II)
- FlowJo v10 (<https://www.flowjo.com/solutions/flowjo/downloads>)

REAGENT SETUP

Cell-suspension buffer

Cell-suspension buffer contains 2% (vol/vol) FBS, 1 mg ml^{−1} poloxamer (P-188), and 100 U ml^{−1} penicillin/streptomycin in calcium- and magnesium-free PBS (CMF-PBS). Filter the buffer through a 0.22- μ m-pore-size membrane. Adjust the pH after preparing the buffer and add NaOH or HCl to bring the pH to ~7.2–7.4. Store the buffer at 4 °C and use within 4 weeks after preparation.

Digestion buffer

Digestion buffer contains 3,000 U ml^{−1} type II collagenase powder, 100 U ml^{−1} DNase, 1 mg ml^{−1} poloxamer 188 (P-188), 1 mg ml^{−1} BSA, 20 mM HEPES buffer, and 1 mM CaCl₂

in Medium 199 with Earle's salts (for digestion in a CO₂ incubator) or in Medium 199 with Hanks' salts (for digestion under atmospheric conditions). Filter the buffer through a 0.22- μ m-pore-size membrane. Adjust the pH after preparing the buffer and add NaOH or HCl to bring the pH to ~7.2–7.4. Store the buffer at 4 °C and use within 1 week after preparation.

Culture medium

The culture medium contains 10% (vol/vol) FBS and 100 U ml⁻¹ penicillin/streptomycin in MEMa medium. Filter the buffer through a 0.22- μ m-pore-size membrane. Store the buffer at 4 °C and use within 4 weeks after preparation.

EQUIPMENT SETUP

Flow cytometer

Ensure that the flow cytometer is equipped with the lasers and filters required to detect the fluorophores. At minimum, a three-laser cytometer with green (488 nm), red (640 nm), and blue (405 nm) lasers is required. The following table summarizes the recommended lasers and filters for optimal results.

Fluorophore	Laser (nm)	Filter (nm)
PE-Cy5	488 or 561	660/20 BP
AF680	640	730/45 BP
APC-Cy7	640	780/60 BP
PE-Cy7	488 or 561	780/60 BP
BV605	405	610/20 BP
PE	488 or 561	582/15 BP
APC	640	670/30 BP
PI	561	610/20 BP

BP, band-pass.

PROCEDURE

Mouse bone dissection • TIMING 20 min per mouse

1. Acquire a suitable number of 8-week-old or older adult C57BL/6 mice for standard adult skeletal cell isolation.

▲ CRITICAL STEP A single adult mouse is sufficient for *in vitro* colony-formation assays. At least five adult mice are needed to acquire enough cells (i.e., 5,000–10,000 cells) for a single *in vivo* transplantation with p-mSSCs, mBCSPs, PCPs, THYs, or BLSPs. At least ten or more adult mice are needed to acquire enough cells (i.e., 5,000–10,000 cells) to transplant 6C3 or HEC stroma. Fewer mice are required when isolating cells from younger mice, due to higher skeletal cell numbers.

2. Euthanize a mouse by overdosing with 100% (vol/vol) CO₂ until at least 1 min after breathing stops and confirm euthanasia by cervical dislocation.
3. Sterilize the dissection area and the external fur of the mouse with 70% (vol/vol) ethanol.
4. Using scissors or a scalpel, cut along the midline ventral skin surface and then along the forelimbs (axilla to forefoot) and hindlimbs (medial thigh to hindfoot). Anatomic images to aid in the identification of the correct cuts to make can be found in Figure 2a,b.
5. Using curved forceps, dislocate the forelimbs and hindlimbs and store them on ice in a 100- × 20-mm dish with cold cell-suspension buffer.

▲ **CRITICAL STEP** To maximize the cell yield, also dissect the spine and pelvis (see Table 1 for cell-number comparison between limbs-only and whole-skeleton digests). If using the spine, remove the spinal cord from the vertebral column, as myelin can clump the cells and result in lower viability (Fig. 2c).

■ **PAUSE POINT** Dissected bones can be stored on ice with cold cell-suspension buffer for a maximum of 2 h.

6. Remove the skeletal muscle and connective tissue by dissecting with a scalpel and gently rolling the tissue between the fingers with a paper towel.

Tissue dissociation • TIMING 2.5 h

7. Transfer the bones to a mortar and add 5 ml of digestion buffer.
8. Crush the bones with a pestle for at least 1 min.
9. Collect the supernatant in a 50-ml conical tube through a 100-μm-pore-size membrane.
10. Repeat Steps 7–9 until the crushed bones are completely white.

▲ **CRITICAL STEP** Mechanical digestion is key to increasing the surface area for downstream chemical digestion with type II collagenase and therefore improving cell yield. Images of bones before and after crushing can be found in Figure 2d,e.

▲ **CRITICAL STEP** During mechanical digestion, prewarm the digestion buffer in a 37 °C water bath for at least 10 min before the next step.

11. After crushing, transfer the minced bones to the same 50-ml collection tube, and add excess digestion buffer until the total volume is a maximum of 15 ml per sample.
12. Incubate the buffer at 37 °C for 30 min at a shaking speed of 210 r.p.m. (for a shaker with a 25-mm orbit diameter) to facilitate tissue digestion.
13. After incubation, filter the digest supernatant through a 100-μm filter into a clean 50-ml conical tube with at least 20 ml of cold cell-suspension buffer.

▲ CRITICAL STEP Filtering removes bone chips and prevents cell clumping. It is important to add sufficient cold cell-suspension buffer to quench the collagenase buffer. We recommend a minimum of 2:1 cold-suspension buffer to digest supernatant ratio.

14. Centrifuge the cell supernatant at 200g for 5 min at 4 °C. Aspirate the supernatant and resuspend the cell pellet in 1 ml of cold cell-suspension buffer and store on ice.
15. Add 15 ml of pre-warmed digestion buffer to the crushed bones and repeat Steps 11–14 two more times (Supplementary Fig. 1). After each subsequent digestion, collect the supernatant in the same 50-ml collection tube used in Step 13.

? TROUBLESHOOTING

Hematopoietic cell depletion • TIMING 2 h

16. Aspirate the supernatant and resuspend the cell pellet in 5 ml of ACK lysis buffer for red blood cell lysis.
17. Incubate the mixture on ice for no longer than 5 min.
18. Quench the ACK lysis solution with 25 ml of cell-suspension buffer.
19. Centrifuge the mixture at 200g for 5 min at 4 °C.
20. Aspirate and discard the supernatant, and resuspend the cell pellet in 5 ml of cell-suspension buffer.
21. Leave the cell suspension on ice for 5 min to allow any remaining bone chips to settle.
22. Transfer the cell suspension to another tube carefully, without disturbing the bone chips.

▲ CRITICAL STEP Bone chips can clog the MACS columns and the flow cytometer, and are a major source of autofluorescent debris. Bone chips readily settle within 5 min while the cells remain in suspension. Use a 1-ml pipettor to remove the first 4 ml, and then use a 200- μ l pipettor to remove the rest. Leave 100–200 μ l behind to minimize the uptake of bone chips.

23. Centrifuge the transferred cell suspension at 200g for 5 min at 4 °C.
24. Resuspend the cell pellet in 1:5 CD45 and 1:10 TER119 Miltenyi Biotec magnetic beads and incubate them for 15 min at 4 °C under gentle agitation.
25. Wash the beads by adding excess cell-suspension buffer.
26. Centrifuge the mixture at 200g for 5 min at 4 °C.
27. Resuspend the cell pellet in 1 ml of cell-suspension buffer.
28. Prepare the mixture for MACS depletion by placing an LD column on a magnet above the collection tube at 4 °C.

29. Rinse the column with 3 ml of cell-suspension buffer.
30. Add 1 ml of sample to the LD column through a 40- μ m-pore-size membrane.
31. Wash the column with 1 ml of cell-suspension buffer twice.
32. Centrifuge the effluent at 200g for 5 min at 4 °C.

? TROUBLESHOOTING

Antibody staining • TIMING 1 h

33. Resuspend the cell pellet in 250 μ l of 20 μ l ml⁻¹ rat IgG and incubate the mixture for 10 min at 4 °C. Rat IgG serves to block endogenous Fc- γ receptors and reduces background during staining.
34. After rat-IgG blocking, transfer 10 μ l of cell solution to each of eight FMO control tubes containing 160 μ l of cell-suspension buffer. Leave the remaining 170 μ l for the fully stained, experimental sample.

▲ CRITICAL STEP An FMO control is one in which every fluorophore-conjugated antibody or fluorescent chemical, except the one of interest, is added to the tube. This identifies the true negative signal for the antibody or chemical of interest and, therefore, informs the gating strategy (Fig. 4).

35. Stain the eight FMO controls and experimental sample based on the antibodies and concentrations provided in the table below for 20 min in the dark on ice.

Antibody	Clone	Fluorophore	Concentration
Anti-CD45	30-F11	PE-Cy5	1:200
Anti-TER119	TER119	PE-Cy5	1:200
Anti-TIE2	TEK4	AF680	1:20
Anti-THY1.1	HIS51	APC-Cy7	1:100
Anti-THY1.2	53-2.1	APC-Cy7	1:100
Anti-CD105	MJ7/18	1° Biotin	1:50
		2° Streptavidin-PE-Cy7	1:100
Anti-CD200	OX-90	BV605	1:50
Anti-ITGAV	RMV-7	PE	1:50
Anti-6C3	6C3	APC	1:100

36. Wash off the primary antibody by adding excess cell-suspension buffer to each tube.
37. Centrifuge the tubes at 200g for 5 min at 4 °C.
38. Resuspend the cells in 100 μ l of 1:100 streptavidin-PE-Cy7 in cell-suspension buffer and incubate for 20 min in the dark on ice.
39. Wash off the secondary antibody by adding excess cell-suspension buffer to each tube.

40. Centrifuge the tubes at $200g$ for 5 min at 4°C , and then resuspend the cells in $300\text{--}500\ \mu\text{l}$ of cell-suspension buffer.

■ **PAUSE POINT** Stained cell samples can be kept on ice for 1–2 h.

41. Strain each sample through a $40\text{-}\mu\text{m}$ filter immediately before running flow cytometry to prevent clogging of the machine.

Flow cytometry • TIMING 2 h

42. Use a $70\text{-}\mu\text{m}$ nozzle and steady the stream.
▲ **CRITICAL STEP** Request the support of a flow-cytometry coordinator if you are unfamiliar with operating the machine.
43. Perform fluorescence compensation to correct for spectral overlap, which may occur when multiple fluorophores with overlapping emission spectra are used. Prepare the tubes with one drop of OneComp eBeads each. Leaving one tube untouched, add single-fluorophore-conjugated antibodies to the remaining tubes. Run the samples individually and calculate the compensation matrix.
44. Calculate drop delay using AccuDrop beads to ensure that the correct droplet will be assigned for sorting.
45. Align the stream to target the center of the collection tube by adjusting the plate voltages.
46. Turn on the chiller and the aerosol-management system to maintain a sterile 4°C environment for sorting.
47. Add $1\ \mu\text{g ml}^{-1}$ of the live-dead dye, PI, to the samples.
48. Run the FMO samples first to establish the gating strategy for sorting. Generate the following panels for optimal results: (i) SSC-A versus FSC-A to capture all cells; (ii) FSC-W versus FSC-H to capture singlets; (iii) SSC-W versus SSC-H to capture singlets; (iv) CD45/TER119 versus PI to capture all live (PI^{-}), non-hematopoietic (CD45^{-}), non-red blood cells (Ter119^{-}); (v) ITGAV versus TIE2 to capture the $\text{TIE2}^{-}\text{ITGAV}^{+}$ population; (vi) THY1 versus 6C3 to capture three populations ($\text{THY1}^{+}\text{6C3}^{-}$, $\text{THY1}^{-}\text{6C3}^{-}$, and $\text{THY1}^{-}\text{6C3}^{+}$); and (vii) each of these populations can be further divided by ITGAV versus CD105, and the $\text{THY1}^{-}\text{6C3}^{-}\text{CD105}^{-}$ and $\text{THY1}^{+}\text{6C3}^{-}\text{CD105}^{+}$ populations can be further divided by ITGAV versus CD200. Examples of typical FACS plots that are obtained are shown in Figures 3b and 4. Expected percentages and cell numbers can also be found in Figure 3b and Table 1.

? TROUBLESHOOTING

49. Sort your desired population of cells into $200\ \mu\text{l}$ of cell-suspension buffer in a collection tube on 'yield' setting.
50. Resort the collected cells into $200\ \mu\text{l}$ of cell-suspension buffer or the desired medium using the 'purity' setting.

▲ **CRITICAL STEP** 5,000–10,000 skeletal cells are needed for *in vivo* engraftment under the renal capsule and 1–500 p-mSSCs are needed for *in vitro* colony-formation assays.

? TROUBLESHOOTING

Downstream functional assays

51. At this point in the protocol, proceed with either *in vivo* renal subcapsular transplantation (option A, see also Figs. 5 and 6), or *in vitro* colony-formation assay (option B, see also Fig. 7) to functionally assess skeletal-lineage cells or alternative experiments relevant to your experimental study.
 - A. *In vivo* renal subcapsular transplant • TIMING 1 h
 - i. After sorting 5,000–10,000 skeletal cells (p-mSSC, mBCSP, PCP, THY, 6C3, BLSP, or HEC), carefully aspirate the supernatant and use a P2 pipette to remove any remaining residue.
 - ii. Resuspend the cell pellet in 2 μ l of Matrigel on ice.

▲ **CRITICAL STEP** This step must be done on ice, as Matrigel thaws at 4 °C and solidifies at – 20 °C and room temperature (23–25 °C).
 - iii. Aspirate 2 μ l of cell-laden Matrigel into the Wiretrol II syringe after assembling the plunger and micropipette as shown in Figure 5 (top). Set the loaded syringes aside at room temperature to let the Matrigel plug solidify.
 - iv. Anesthetize 8- to 12-week-old adult immunodeficient Rag2/ γ (c)KO mice using inhaled isoflurane gas or i.p. injected tribromoethanol. To use isoflurane gas, place the mouse into an anesthesia chamber, and deliver 2 liters min^{-1} of 2% (vol/vol) isoflurane in 100% (vol/vol) oxygen. Check for righting or paw-withdrawal reflex to ensure that the mouse is unconscious. Transfer the mouse to a homeothermic blanket and place its snout into an anesthesia nose cone delivering 2–3 liters min^{-1} of 2% isoflurane in 100% (vol/vol) oxygen. Monitor the mouse's respiratory rate and adjust the isoflurane concentration as needed. To use tribromoethanol, weigh the mouse and inject 250 mg kg^{-1} tribromoethanol i.p. Induction takes 1–2 min and anesthesia lasts for 40–90 min. Check for righting or paw-withdrawal reflex to ensure that the mouse is unconscious. Use sterile needles for injection.
 - v. Administer 1 mg kg^{-1} buprenorphine SR s.c.

▲ **CRITICAL STEP** Providing analgesia before surgery results in superior pain relief.

- vi. Prepare the midline dorsal skin with 70% (vol/vol) ethanol and povidone-iodine solution. Drape the mouse with sterile drapes, leaving the surgical site exposed.
- vii. Palpate for the kidneys under the skin (feel for a jelly-bean-like shape) and then, using scissors and forceps, make a single 1-cm incision over the kidneys. Use blunt dissection to separate the skin from the abdominal wall.
- viii. Make a < 1-cm incision into the abdominal wall in the direction of the fibers of the external oblique muscle above the kidney and gently protrude the kidney through the cut by massaging the surrounding abdominal wall.

▲ CRITICAL STEP Use only blunt tools to resect the tissues. Palpating for the kidneys before incision is a critical step to ensure that the kidney is the first organ to protrude from the incision.

- ix. Identify the kidney poles and make a 1- to 2-mm shallow incision with a 31-gauge needle into the renal capsule (Fig. 5, center left). Make sure to use sterile needles and cut with the bevel up.

▲ CRITICAL STEP Cutting at the kidney poles rather than near the middle is critical to avoiding rupture of the capsule and ensuring adequate space for the transplant. In the case of bleeding, flush the tissue with warm sterile PBS.

- x. Using a pipette tip, gently separate the capsule from the underlying renal parenchyma, being careful not to tear the capsule.
- xi. Insert the loaded syringe under the renal capsule and push the plunger to eject the Matrigel plug into the capsule pocket (Fig. 5, center right).

? TROUBLESHOOTING

- xii. Using forceps, place a piece of sponge under the capsule to prevent the plug from falling out.
- xiii. Add 1 ml of warm PBS to the abdominal cavity to prevent postoperative dehydration.
- xiv. Close the abdominal wall with 6–0 simple interrupted sutures.
- xv. Close the skin with 6–0 simple interrupted sutures or staples.
- xvi. Transfer the mice to a new cage with a homeothermic blanket and antibiotic water, and follow institutional protocols for postoperative care.

- xvii. Wait for at least 4 weeks before graft explant and downstream analysis (Figs. 5 (bottom) and 6).

? TROUBLESHOOTING

B. *In vitro* colony formation • TIMING 2 weeks

- i. After sorting between 1 and 500 p-mSSCs or mBCSPSs into culture medium (instructions for medium preparation are available in MATERIALS: Reagent Setup), transfer the contents of the entire tube to a 0.1% (wt/vol) gelatin-coated 100- × 20-mm dish with 10 ml of culture medium. Swirl the plate gently to evenly distribute the cells throughout the plate.

▲ critical step To coat plates for tissue culture, add 5 ml of 0.1% (wt/vol) gelatin in sterile water and incubate the plates at room temperature for 1 h. Aspirate the 0.1% (wt/vol) gelatin immediately before culturing the cells.
- ii. Incubate the plate at 37 °C under low O₂ (2% (vol/vol) atmospheric oxygen, 7.5% (vol/vol) CO₂) conditions.
- iii. Replace the medium with fresh medium after 7 d of incubation.
- iv. Count the colonies under a light microscope after 14 d of incubation (Fig. 7a,b).

▲ critical step At this point, colonies can be fixed and analyzed by immunohistochemistry, alizarin red S, or alcian blue (Fig 7c,d), or they can be prepared for FACS analysis as described in the following steps.

? TROUBLESHOOTING

- v. To prepare colonies for FACS analysis, carefully aspirate the culture medium and gently wash the plates with PBS.
- vi. Using vacuum silicone grease, attach the cloning rings around each colony.

▲ critical step The cloning rings can be prepared in-house from the mouths of Eppendorf tubes of various volumes (depending on the diameter of the colonies). First, cut off the cap from where it joins the lip of the mouth with scissors. Then cut the mouth from the rest of the tube with fine scissors to form a cylindrical well. Next, apply vacuum silicone grease to the lip of the tube, and attach the cloning ring to the colony's perimeter immediately after the culture medium is aspirated. Take special care to prevent desiccation of the cells during this step.

- vii. Add 100 μ l of 3,000 U ml⁻¹ type II collagenase to each ring.
- viii. Incubate the plate for 10 min at 37 °C.
- ix. Collect cells from each ring and transfer them to a fresh Eppendorf tube with at least 1 ml of cell-suspension buffer.
- x. Wash the ring with 100 μ l of cell-suspension buffer to collect the remaining cells and add them to the respective Eppendorf tube.
- xi. Carry out antibody staining and flow-cytometry analysis as described in Steps 33–48.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 2.

• TIMING

Steps 1–6, mouse bone dissection: 20 min per mouse

Steps 7–15, tissue dissociation by mechanical and chemical digestion: 2.5 h

Steps 16–32, hematopoietic cell depletion by ACK lysis and MACS: 2 h

Steps 33–41, primary and secondary antibody staining: 1 h

Steps 42–50, flow cytometry, including setup and sorting: 2 h

Step 51A, *in vivo* renal subcapsular transplantation, including preoperative setup and surgery: 1 h

Step 51B, *in vitro* colony-formation assay, including plating, culture, and lifting of the cells: 2 weeks

ANTICIPATED RESULTS

The best measure of a successful isolation is the presence of viable skeletal-cell populations on a flow cytometer. Cell viability, as measured by the percentage of trypan-blue-negative cells, should be at least 85%. If cell viability is below 70%, use Table 2 to improve the isolation. PI can also serve as a viability marker on the flow cytometer, but the percentage of live cells is underestimated due to the presence of auto-fluorescent PI⁺ bone chips and debris. Despite CD45 and TER119 depletion, there will still be many cells staining positive for CD45 and TER119, so it is important to use FACS gating to exclude these cells in the gating scheme for skeletal cells. The representative FACS plots and the expected percentage and number of cells from a single 8-week C57BL/6 mouse can be found in Figure 3b and Table 1. Gating should be based on FMO controls for each color; the gating applied for Figure 3b and Table 1 is shown in Figure 4.

Representative gross and GFP fluorescence images of a skeletal graft 4 weeks after renal subcapsular transplantation of 10,000 p-mSSCs from an 8-week-old C57BL/6-Tg(CAG-EGFP)10sb/J mouse can be found in Figure 5. The Movat pentachrome staining of skeletal grafts generated 4 weeks after transplantation of various skeletal subpopulations is shown in Figure 6. For example, the PCPs (CD45⁻TER119⁻TIE2⁻ITGAV⁺THY1⁺6C3⁻CD105⁺CD200⁺) give rise to only cartilage (blue); the osteoprogenitors, which include THYs (CD45⁻TER119⁻TIE2⁻ITGAV⁺THY1⁺6C3⁻CD105⁺CD200⁻) and BLSPs (CD45⁻TER119⁻TIE2⁻ITGAV⁺THY1⁺6C3⁻CD105⁻), give rise to only bone (orange); the stromal cells, which include 6C3s (CD45⁻TER119⁻TIE2⁻ITGAV⁺THY1⁻6C3⁺CD105⁺) and HECs (CD45⁻TER119⁻TIE2⁻ITGAV⁺THY1⁻6C3⁺CD105⁻), give rise to bone (orange) and marrow-supportive cells (red); and the mBCSPs (CD45⁻TER119⁻TIE2⁻ITGAV⁺THY1⁻6C3⁻CD105⁺) and p-mSSCs (CD45⁻TER119⁻TIE2⁻ITGAV⁺THY1⁻6C3⁻CD105⁻) give rise to all three lineages (cartilage, bone, and stroma). Skeletal grafts can be dissociated and re-analyzed for skeletal cells, which in the case of p-mSSC grafts should include self-renewed p-mSSCs, downstream mBCSPs, and individual subpopulations from each of the three lineages. Host hematopoietic, endothelial, and adipocytic cells often integrate with the ectopic grafts and can be identified by histology or isolated by flow cytometry.

Finally, representative light-microscopy images from *in vitro* colony-formation assays, in which 500 p-mSSCs from 8-week C57BL/6 mice were plated into culture media, can be found in Figure 7a,b. An example of alizarin red S staining for osteogenic potential and alcian blue staining for chondrogenic potential can be found in Figure 7c and d, respectively. Single p-mSSCs and mBCSPs are capable of giving rise to multilineage colonies. Dissociation and re-analysis of colonies from p-mSSCs by flow cytometry will demonstrate the presence of self-renewed p-mSSCs, downstream mBCSPs, and individual subpopulations from each of the three lineages.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1 |.

Overview of the protocol for skeletal stem cell lineage isolation and functional assessment. The protocol includes isolation of the mouse skeleton by tissue dissection (Steps 1–6); dissociation of the skeleton into a single-cell suspension by mechanical (Steps 7–10) and chemical digestion (Steps 11–15); depletion of red blood cells with ACK lysis buffer (Steps 16–23) and magnetic-activated cell sorting (MACS) (Steps 24–32); antibody-staining of the cells for skeletal lineage surface markers (Steps 33–41) and analysis and sorting of the cells on a flow cytometer (Steps 42–50); functional assessment of skeletal-lineage phenotype by *in vivo* renal subcapsular transplantation (Step 51A) and *in vitro* colony-formation assay (Step 51B); analysis of grafts by Movat pentachrome and immunohistochemistry (left), and analysis of cultures by immunohistochemistry, alcian blue staining, or alizarin red S staining (right).

Figure 2 |.

Schema for mechanical digestion of skeletal tissue. **(a)** The mouse is mounted on a platform after euthanasia, and the surgical site is sterilized with 70% (vol/vol) ethanol (Steps 2 and 3). **(b)** The skin is cut down the ventral and dorsal midline, and along the limbs to expose the underlying skeletal structures. H (humerus), P (pelvis), F (femur), T (tibia), and V (vertebrae) are dissected (Steps 4 and 5). **(c)** The bones are cleaned by dissection with a scalpel and gentle rolling of the tissue between the fingers with paper towels (Step 6). **(d)** Bones are placed in 5 ml of digestion buffer (Step 7). **(e)** Bones are crushed with mortar and pestle (Step 8). (Inset) The bones after three rounds of crushing with mortar and pestle in digestion buffer. All animal experiments in this figure were performed in accordance with the Stanford Administrative Panel on Laboratory Animal Care (APLAC) and received approval from the Institutional Review Board (IRB).

Figure 3 |.

Skeletal stem cell lineage hierarchy and flow cytometry gating scheme. (a) Skeletal stem cell lineage hierarchy of a multipotent, self-renewing mouse skeletal stem cell (mSSC) that gives rise to an early progenitor (pre-mBCSP) and a late progenitor, the mouse bone, cartilage, and stromal progenitor (mBCSP), which in turn generates a pro-chondrogenic progenitor (PCP), two distinct osteoprogenitors, the THY population and the BLSP (B-cell lymphocyte-stimulating population), and two distinct stroma, the 6C3 and HEC (hepatic leukemia-factor-expressing cell) populations. THY and 6C3 are hematopoiesis-supportive,

whereas BLSPs are B-cell promoting. mSSCs and pre-mBCSPs are collectively referred to as phenotypic mSSCs (p-mSSCs). **(b)** Gating strategy recommended to sort the different cells of the skeletal stem cell lineage as shown in **a** (Steps 42–50). Representative FACS plots with the percentage of parent gate for each population, as generated on the BD FACS Aria II and analyzed by FlowJo v10, are shown. Color code for FACS gates: dark gray: forward scatter, side scatter, viability, and lineage gates; green: osteochondral lineage gate; orange: osteo-lineage cells, including BLSP and THY; blue: chondro-lineage cells, including PCPs; red: stroma, or 6C3 and HEC; and purple: stem and progenitors, including mBCSPs, p-mSSCs, pre-mBCSPs, and mSSCs. A, area; AF, Alexa Fluor; APC, allophycocyanin; BV, brilliant violet; FSC, forward scatter; H, height; PE, phycoerythrin; PI, propidium iodide; SSC, side scatter; W, width. All animal experiments in this figure were performed in accordance with the Stanford APLAC and received approval from the IRB.

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Figure 4 |.

Fluorescence-minus-one (FMO) controls for mouse skeletal lineage gating strategy.

Representative FACS plots as generated on the BD FACS Aria II and analyzed by FlowJo v10 for each FMO control (e.g., anti-CD45/TER119 in PE-Cy5, anti-TIE2 in Alexa Fluor 680, anti-6C3 in Alexa Fluor 647, anti-CD105 in PE-Cy7, anti-ITGAV in PE, anti-THY1.1/THY1.2 in APC-Cy7, anti-CD200 in BV605, and propidium iodide) are shown (Steps 34, 35 and 43). FMO controls identify the true negative signal for a given antibody or chemical of interest, and therefore inform the gating strategy. Representative FACS plots with the percentage of parent gate are shown for each population. All animal experiments in this figure were performed in accordance with the Stanford APLAC and received approval from the IRB.

Figure 5 |.

Schema for renal subcapsular transplantation of skeletal-lineage cells. (Top) Cells are resuspended in 2 μ l of Matrigel on ice and then aspirated into a Wiretrol II syringe after assembly of the micropipette and plunger (Step 51A(ii and iii)). (Center left) A superficial incision is made near the kidney pole to separate the capsule from the renal parenchyma (Step 51A(ix)). (Center right) Cell-laden Matrigel is injected into the capsule pocket by gently pushing on the plunger (Step 51A(xi)). (Bottom) Heterotopic ossicles form ~4 weeks after transplantation (Step 51A(xvii)). Upper-left panel: Gross image of the kidney graft 4 weeks after 10,000 p-mSSCs from C57BL/6-Tg(CAG-EGFP)10sb/J mice were transplanted under the renal capsule. Scale bar, 5 mm. Upper-right panel: High magnification of the gross image. Scale bar, 2 mm. Lower-left panel: GFP fluorescence of the same kidney graft. Scale

bar, 5 mm. Lower-right panel: High magnification of the GFP fluorescence image. Scale bar, 2 mm.

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Figure 6 |.

Movat pentachrome staining of renal subcapsular grafts. Movat pentachrome stains of renal subcapsular grafts 4 weeks after transplantation of 10,000 of each skeletal-cell population are shown on the right. Movat pentachrome staining was carried out as described in refs. 32,36. Scale bars, 200 μ m. Color code for FACS gates and stains: green: osteochondral lineage; orange: osteo-lineage cells, including BLSP and THY; blue: chondro-lineage cells, including PCPs; red: stroma, or 6C3 and HEC; and purple: stem and progenitor cells, including mBCSPs, p-mSSCs, pre-mBCSPs, and mSSCs. All animal experiments in this figure were performed in accordance with the Stanford APLAC and received approval from the IRB.

Figure 7 |.

In vitro colony-formation assays with p-mSSCs. **(a-d)** The expected results from Step 51B.

(a) Low-magnification (4×) light-microscopy image of colonies generated 2 weeks after plating 500 p-mSSCs. Scale bar, 500 μm. **(b)** High-magnification (10×) light-microscopy image of colonies generated 2 weeks after plating 500 p-mSSCs. Scale bar, 100 μm. **(c)** Light-microscopy images of alizarin red S staining of colonies generated 2 weeks after plating 500 p-mSSCs, demonstrating osteogenic potential. Scale bar, 100 μm. **(d)** Light-microscopy images of alcian blue staining of colonies generated 2 weeks after plating 500 p-mSSCs, demonstrating osteogenic potential. Scale bar, 100 μm. Alizarin red S and alcian blue staining was carried out as described in ref. 14. All animal experiments in this figure were performed in accordance with the Stanford APLAC and received approval from the IRB.

Yields for the protocol.

Table 1 |

Cell populations	Estimated total cell no., limbs only (<i>n</i> = 3) (average ± s.d.)	Estimated total cell no., whole skeleton (<i>n</i> = 2) (average ± s.d.)
All cells	104,104 ± 32,852	144,601 ± 30,032
Single cells by FSC	84,052 ± 24,784	127,944 ± 20,574
Single cells by SSC	78,359 ± 22,537	124,739 ± 16,394
CD45 ⁺ TER119 ⁺ PI ⁻	12,110 ± 2,883	35,610 ± 20,280
ITGAV ⁺ TIE2 ⁻	6,243 ± 673	12,052 ± 4,228
THY1 ⁺ 6C3 ⁻	2,145 ± 231	4,211 ± 1,972
CD105 ⁺	1,249 ± 358	2,854 ± 1,658
CD200 ⁺ (PCP)	464 ± 99	1,187 ± 654
CD200 ⁺ (THY)	716 ± 262	1,390 ± 715
CD105 ⁺ (BLSP)	478 ± 231	1,158 ± 179
THY1 ⁺ 6C3 ⁺	525 ± 101	780 ± 56
CD105 ⁺ (6C3)	339 ± 67	582 ± 58
CD105 ⁺ (HEC)	173 ± 42	179 ± 0
THY1 ⁺ 6C3 ⁻	2,588 ± 73	5,482 ± 2,385
CD105 ⁺ (mBCSP)	1,477 ± 22	3,870 ± 2,143
CD105 ⁺ (p-mSSC)	999 ± 54	1,481 ± 248
CD200 ⁺ (mSSC)	665 ± 55	779 ± 60
CD200 ⁺ (pre-mBCSP)	269 ± 29	603 ± 266

The estimated total number of cells obtained from each C57BL/6 mouse is given in the supplementary Data. Green, osteochondral lineage; blue, chondro-lineage; orange, osteo-lineage; red, stromal lineage; purple, stem cells and progenitors.

Table 2 |

Troubleshooting table.

Steps	Problem	Possible reason(s)	Possible solution(s)
15	Low yield of cells after chemical digestion (expected yields from crushed bones before and after each digest can be found in supplementary Fig. 1)	(i) Digestion buffer pH is too acidic or alkaline, resulting in cell death (ii) Inadequate mechanical digestion in the previous steps (iii) Temperature and shaker speed were not set to 37 °C and 210 r.p.m., respectively	(i) Adjust the digestion buffer pH to ~7.2–7.4 by adding NaOH or HCl or increasing HEPES buffer concentration (iii) Crush the bones with mortar and pestle until the bones are completely white. In addition, mince the bones with razor blades (iii) Set the shaker to 37 °C and at 210 r.p.m. for a 25-mm-diameter orbital shaker. Type II collagenase and DNase work optimally in these conditions
32	Low yield of cells after MACS	(i) LD columns are clogged	(i) Remove the spinal cord when including vertebrae, as myelin can increase clumping (Step 5) (ii) Repeat Steps 21 and 22 to remove bone chips (iii) Resuspend the sample in more than the suggested 1 ml in Step 27 to dilute cell concentration (iv) Strain the sample through a 40-µm-pore-size membrane
48	Dim signal for CD200 in BV605	(i) Low surface expression (ii) Poor antibody binding	(i) Instead of the CD200 BV605 antibody, perform a two-step stain with purified rat anti-mouse CD200 (clone OX-90) and goat anti-rat Qdot 605 before starting the staining procedure in Step 35
50	Low yield of cells after flow cytometry	(i) Use of different antibody clone (ii) Improper flow cytometer setup	(i) Make sure to use the same antibody clones as those listed in the table at Step 35 and the Reagents section (ii) Work with a FACS expert to set up the flow cytometer
51A(xi)	Difficulty in obtaining Matrigel plugs under a renal capsule	(i) The kidney is too small (ii) The subcapsular pocket made by the incision is too narrow or too wide	(i) Use 10-to-12-week-old male mice with larger kidneys (ii) Make incisions at the kidney poles; use a surgical sponge to secure the Matrigel plugs in the pocket
51A(xvii)	No graft at 4 weeks post transplantation	(i) Matrigel plugs slipped out (ii) Fewer than 5,000 skeletal cells were transplanted	(i) Place Matrigel deep in the pocket, and use a surgical sponge to secure the Matrigel plugs in the pocket (ii) Extract more bones; see troubleshooting advice for low cell yield in different steps of the protocol; confirm the cell-gating scheme and sort settings on the flow cytometer
51B(iv)	No colonies 2 weeks post FACS sorting	(i) Poor culture conditions (ii) p-mSSCs or mBCSPs were not sorted	(i) Use fresh culture medium; clean the incubator, and use sterile technique (ii) Confirm p-mSSC or mBCSP gating scheme and sort settings on the flow cytometer