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Purification and functional characterization of novel human skeletal stem cell lineages

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Author contributions

C.K.F.C., T.H.A., M.T.L. and R.S. conceived the isolation strategy and functional assays. C.K.F.C. and M.T.L. supervised the project. T.H.A., M.Y.H, H.M.S., L.S.K., M.P.M. and Y.W. developed the protocol, performed the experiments and analyzed the data. M.Y.H. wrote the manuscript. A.A.A., E.J.A., L.Z., M.G.K.B., E.T. and S.P.S. assisted with flow cytometry, in vitro assays and manuscript preparation. J.B., M.G., S.H. and S.G. provided clinical skeletal specimens and edited the manuscript. D.S., R.S., M.M. and N.N. assisted in the bioinformatics and single-cell sequencing platforms

Competing interests

The authors declare no competing interests.

Additional information

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Abstract

Human skeletal stem cells (hSSCs) hold tremendous therapeutic potential for developing new clinical strategies to effectively combat congenital and age-related musculoskeletal disorders. Unfortunately, refined methodologies for the proper isolation of bona fide hSSCs and the development of functional assays that accurately recapitulate their physiology within the skeleton have been lacking. Bone marrow-derived mesenchymal stromal cells (BMSCs), commonly used to describe the source of precursors for osteoblasts, chondrocytes, adipocytes and stroma, have held great promise as the basis of various approaches for cell therapy. However, the reproducibility and clinical efficacy of these attempts have been obscured by the heterogeneous nature of BMSCs due to their isolation by plastic adherence techniques. To address these limitations, our group has refined the purity of individual progenitor populations that are encompassed by BMSCs by identifying defined populations of bona fide hSSCs and their downstream progenitors that strictly give rise to skeletally restricted cell lineages. Here, we describe an advanced flow cytometric approach that utilizes an extensive panel of eight cell surface markers to define hSSCs; bone, cartilage and stromal progenitors; and more differentiated unipotent subtypes, including an osteogenic subset and three chondroprogenitors. We provide detailed instructions for the FACS-based isolation of hSSCs from various tissue sources, in vitro and in vivo skeletogenic functional assays, human xenograft mouse models and single-cell RNA sequencing analysis. This application of hSSC isolation can be performed by any researcher with basic skills in biology and flow cytometry within 1–2 days. The downstream functional assays can be performed within a range of 1–2 months.

Introduction

The human skeletal system is a collective network of diverse tissue types including bone, cartilage, fat, fibroblasts, endothelium, nerves and hematopoietic cells maintained by populations of tissue-specific adult stem cells[1–3]. Stem cells are known to play vital roles in tissue development, homeostasis and regeneration, and have been well characterized in skeletal niches such as the hematopoietic stem cell lineage[4–6]. Unfortunately, stem cell regulation and the hierarchical organization of human skeletal progenitors in nonhematopoietic lineages, including bone and cartilage, has been largely unexplored. This is partly due to the absence of reliable cell-surface markers, tissue dissociation protocols and functional stemness assays. Early isolation efforts relied on the ability of bone marrow-derived cells to adhere to plastic dishes, however, these ‘mesenchymal stromal/stem cells’ (‘MSCs’) contain heterogeneous mixtures of cells with indeterminate potencies and promiscuous contribution to many overlapping lineages, such as bone, cartilage, fat, muscle, fibroblast, endothelial cells and stroma[7–11]. Throughout the years, the characterization of bone marrow-derived MSCs (BMSCs) have been refined by the identification of several specific cell surface markers, but their multipotency and self-renewal output remains highly variable[12–14]. Importantly, the skeletal research field suffers

from a lack of standardized methodology to isolate BMSCs, which has contributed to the heterogeneity across laboratories, begging the need for the current work.

Using lineage-tracing and clonal analysis techniques, we have reported the existence of skeletal stem cells (SSCs) in mice that exclusively give rise to bone, cartilage and hematopoietic-supporting stroma[15]. We have furthermore identified and characterized the analogous human SSC (hSSC) that displays distinct cell surface markers from its mouse counterpart[16]. Here, we provide a suite of recently developed protocols that will enable researchers to purify and study hSSCs from fetal, adult bone, fracture callus and induced pluripotent stem cell (iPSC) specimens for further exploration of hSSCs in the context of bone development, regeneration, aging and disease (Fig. 1 and Extended Data Fig. 1). The initial protocol will describe the mechanical and enzymatic dissociation of bone from various tissue sources to extract viable cells and the subsequent flow cytometric sorting based on our reported cell surface markers for human SSCs. We will also detail in vivo and in vitro assays for assessing the functional output of the isolated cells, which includes the renal capsule and human xenograft subcutaneous models.

Isolation of the hSSCs capable of multilineage differentiation

Compared with postnatal bone, fetal human skeletal elements (17–20 gestational weeks old) are highly enriched for populations of skeletogenic progenitors, which increases the likelihood of isolating rare skeletal stem/progenitor populations. Moreover, at this point, the skeletal matrix is less mineralized, which aids in cellular extraction, altogether providing the ideal prerequisite for detailed interrogation into prospectively purified cell types at this developmental stage. On the basis of our previous identification of the analogous mouse skeletal stem cell and the characterization of its lineage tree, we employed a comparative approach leveraging functional and transcriptomic assays on zonally micro-dissected human fetal growth plate to identify corresponding human skeletal lineage populations[15,16]. We have arrived at a profile that via fluorescence-activated cell sorting (FACS) gates out hematopoietic lineages ($CD45^+CD235^+$), as well as perivascular and endothelial lineages ($CD31^+$, $TIE2^+$). We used single-cell RNA sequencing (scRNAseq) and our previous panel of mouse skeletal stem cell markers to identify two subsets of $CD45^-CD235a^-$ (nonhematopoietic) and $CD31^-TIE2^-$ (nonendothelial, perivascular) cells in the growth plate of fetal femurs, $Podoplanin\ (PDPN)^+CD146^-CD73^+CD164^+$ and $PDPN^+CD146^{low}CD73^+CD164^+$, that were multipotent and capable of forming ossicles in vivo through endochondral ossification[15,16]. These populations can be differentiated by FACS gating for PDPN and CD146, consequently delineating the two cell populations that are $PDPN^+$ but vary in their lacking or low expression of CD146.

Serial transplantation in vivo assays revealed the $PDPN^+CD146^-CD73^+CD164^+$ subset to be self-renewing, multipotent skeletal stem cells that gave rise to all the other populations —hence termed the hSSC[16]. Sequentially, we found the $PDPN^+CD146^+$ subset to be downstream of the hSSC, and termed these cells human bone, cartilage and stroma progenitors (hBCSPs). We also identified a further five cell subsets, which showed defined unipotency in functional transplantation assays and divide into two lineages: (1) two osteogenic subsets, a $PDPN^-CD146^+THY1^{high}$ and a $PDPN^-CD146^+THY1^{low}$

population—collectively referred to as the human osteoprogenitors (hOPs) and (2) three chondrogenic subsets, a $PDPN^{+}$ $CD146^{-}CD73^{-}CD164^{+}$, a $PDPN^{+}CD146^{-}D73^{+}CD164^{-}$ and a $PDPN^{+}CD146^{-}CD73^{-}CD164^{-}$ population—collectively referred to as the human chondroprogenitors (hCPs)[16].

Other groups have applied immunophenotypic markers to label human skeletal progenitor cells—THY1 (CD90), ENG (CD105), NT5E (CD73) and CD44 (refs. [17–19]). Of note, rather than for the prospective isolation of cell populations, these markers are often used to confirm a putative MSC identity in plastic-adhered, cultured cells, which show artificial skewing of marker profiles. Additional markers such as NGFR (CD271) and MCAM (CD146) have been used to select for cells with higher colony-forming ability and capacity to differentiate into multiple skeletal lineages[20,21]. However, these markers are often used individually as pan-MSC labels and are not specific to hSSCs or cells at anatomical sites of active bone growth (growth plate, periosteum). Moreover, these populations remain very heterogeneous in terms of function and lineage potential[3,22–27]. The novel markers that we have identified, including PDPN and CD164, help to resolve these populations to reveal a specific hSSC followed by defined downstream lineages from tissue-spanning BMSC populations. In addition, our protocol includes a series of mechanical and enzymatical digestion steps to expand all these cell types that can be isolated from skeletal tissues, as they are designed to optimize extraction and recovery of both immature and lineage-restricted skeletal lineages.

Advantages

So far, BMSCs have been considered the primary precursor source for osteoblasts, chondrocytes and stromal cells, and have been used in human clinical trials for almost any imaginable condition spanning from bone fracture healing, spinal cord injuries and osteoarthritis to Alzheimer's disease[9]. Historically, BMSCs were isolated by their plastic adherence to cell culture plates and contained a heterogeneous mixture of cells that indiscriminately contributed to several overlapping lineages[7,10]. Plate adherence techniques were the first approaches used to identify BMSCs, also allowing the isolation of single-cell-derived clones, but these approaches are prone to the introduction of culture artifacts, proliferative exhaustion and altered phenotypes. Throughout the years, BMSCs have been further characterized by the emergence of novel cell surface markers, albeit often retrospectively, large RNAseq datasets and functional assays[12–14]. However, the lack of a standardized method for BMSC isolation has contributed to cellular and functional heterogeneity across laboratories.

Our improved isolation methods have allowed a granular analysis of this cell population, and have produced a much more homogeneous subpopulation of hSSCs with specific downstream lineages that will help to reveal new biology and opportunities for intervention. Our hSSC isolation method is designed to extract intact cells directly from tissue samples without culture, while enriching for specific skeletal progenitor populations on the basis of distinct cell surface marker expression and flow cytometric techniques. FACS-purified hSSCs also represent a refined stem cell population that strictly gives rise to bone, cartilage and stromal cell types. This purity is reflected in the transcriptomic homogeneity of hSSCs

on the single-cell level, as well as the seven skeletal cell subsets of the hSSC lineage that are distinct in their configuration of cell surface markers[16].

These subtypes can be further characterized in terms of their functional activity, including lineage commitment to bone and cartilage, as well as stromal cells that crosstalk with other bone marrow stem cell lineages, such as the hematopoietic stem cell lineage[16]. The ability to resolve individual types of stem and progenitor cells in tissues facilitates a more precise analysis of the regenerative processes in bone tissues and how their dysregulation could result in skeletal disease and aging. Importantly, our here-presented protocol provides a standardized method to isolate and characterize hSSCs and, downstream, defined skeletal lineages across multiple types of sources including fetal bone, fracture callus tissue, adult femoral head specimens and iPSCs.

Lastly, as in vitro assays may be limited in their ability to recapitulate the native physiology of the bone microenvironment, we have also established human xenograft models to study hSSC activity in vivo[28]. These human xenograft models are also amenable to high-throughput genetic and chemical manipulation for the evaluation of hSSCs and downstream progenitors in response to injury and extrinsic cues such as growth factors and small molecule drug compounds. Subsequently, the skeletogenic activity of labeled hSSC can be determined with sophisticated methods including scRNAseq, microcomputed tomography (micro CT), histomorphometry and immunohistochemistry.

Overview of the procedure

To isolate highly purified hSSCs that are able to form bone, cartilage and hematopoiesis-supporting stromal but not fat tissues, we utilize a combination of cell surface markers with flow cytometry (Fig. 1). hSSCs can be found in several skeletal sources, including fetal bone, adult femoral head, adult callus and iPSCs (Fig. 2 and Extended Data Fig. 1). It is important to note that accessibility and legal/ethical consent to obtain some of these human tissues will differ amongst universities and local laws.

In brief, freshly attained human tissue specimens are processed by surgical dissection, mechanical dissociation and finally enzymatic digestion (Steps 1–3). We then use our extensive panel of eight cell surface markers to isolate hSSCs and their downstream lineages via flow cytometry (Steps 4–22). We also outline several functional and molecular downstream assays to assess hSSC stemness. These include renal capsule transplantation (Step 23A), in vitro colony formation and skeletal differentiation assays (Step 23B–D), and SmartSeq2-based scRNAseq (Step 23E). We also describe our in vivo xenograft and microfracture model, used to study hSSCs within their actual niche by transplanting human fetal phalanges subcutaneously in immunodeficient mouse pups (Box 1). Finally, we describe steps to label hSSCs through lentiviral transduction (Box 2).

Applications

The functionality of the skeletal system is multifaceted and is essential for dictating human locomotion, supporting hematopoiesis, protecting the spinal cord of the peripheral nervous system and other several internal organs, as well as providing a niche for many cell types, including local and metastatic cancers[29]. As this vast range of cell types can be recapitulated

in vivo using our xenograft model with complete reconstitution of human hematopoietic stem cell (HSC) niche, the isolation and functional assessment of the hSSCs and its downstream progeny can be broadly utilized for various studies beyond bone and cartilage biology, including the following:

- The intrinsic and extrinsic mechanisms of stem cell self-renewal and multilineage differentiation in the bone as it pertains to forming and shaping skeletal tissue
- The role of skeletal stem cells, progenitors and progeny in physiological processes of bone growth, development and homeostasis, injury and repair, and aging
- The crosstalk of the skeletal stem cell lineage with the immune system
- The role of skeletal stem cells, progenitors and progeny in disease such as bone cancers, like osteosarcoma and chondrosarcoma; degenerative disorders such as osteoarthritis and intervertebral disc degeneration; inherited osteochondral disorders, such as Klippel Feil syndrome, Ankylosing spondylitis and osteochondritis dissecans; their crosstalk with hematopoietic cell type in mediating hematological disorders, such multiple myeloma, Hodgkin's lymphoma and leukemia; and metastatic cancers of the bone, such as breast and prostate cancer

These future studies will provide new opportunities for clinical application of hSSC therapy including isolation and transplantation of purified hSSCs from a variety of sources, as well as endogenous modulation of resident hSSC activity in situ with defined factors.

We have previously demonstrated that in both mouse and human, SSCs and their immediate downstream progenitor, BCSPs, are amplified during fracture healing; however, the frequency of SSCs progressively decreases with age[15,16,28,30]. We also used our preclinical human xenograft model to evaluate if the addition of BMP2 and soluble VEGFR1 can potentiate articular cartilage regeneration.

Indeed, our results found that the addition of these growth factors following an acute injury could stimulate de novo cartilage growth compared with control[28].

In regard to aging, we found that mouse and human SSCs demonstrate intrinsic functional defects in their osteochondrogenic potential in older individuals. Aged mouse SSCs also generate more proinflammatory stroma, driving enhanced bone resorption by osteoclasts locally and 'inflammaging' systemically[31]. In humans, transcriptomic comparison of hSSCs from young and aged patients, isolated using the presented protocol here, revealed downregulation of skeletogenic pathways such as WNT (wingless/integrated) and upregulation of senescence-related pathways in young versus aged hSSCs[32]. Specifically, we identified Sirtuin1, an NAD (nicotinamide adenine dinucleotide)-dependent deacetylase that plays a role in cell metabolism as well as senescence, is repressed in aged hSSCs yet re-activation by trans-Resveratrol or a small-molecule compound dramatically restored in vitro differentiation potential. Interestingly, we found the age-related fracture healing impairment was exacerbated in female callus specimens compared with men. While advanced age

in male-derived hSSCs did not alter CFU-F (colony forming unit fibroblast) capability, female hSSCs displayed a significant decline, which may explain the higher rate of fragility fractures and increased complications during recovery in postmenopausal women relative to men. This also points at direct effects of dimorphic hSSC regulation by sex hormones.

Finally, the specification of the mouse and human skeletal stem cell lineages, using methods described in this protocol, has facilitated the possibility to probe translational potential of findings in the murine model as well as the elucidation of species-specific dichotomies[15,33,34]. In the future, the breadth of questions addressable using purified hSSCs span identification of novel subsets of hSSC and defined skeletal progenitors, SSC-specific genetic and epigenetic pathways behind age-related differences in bone or cartilage regeneration, oncogenic events in SSC leading to neoplasia, crosstalk between SSC and other hematopoietic and endothelial stem cells in bone marrow cell niches, and functional SSC diversity during skeletal development and regeneration[31,32,35].

Limitations

The protocols we describe here present a highly reproducible and standardized discovery platform for isolating functionally defined hSSC and downstream skeletal progenitors for preclinical and future clinical studies. However, the current panel of cell surface markers we present are by no means comprehensive and there are probably additional hSSC subtypes and downstream lineage-restricted progenitors that could be further resolved by expanding the number of surface markers employed that could be identified using some of the techniques we describe here and beyond. On the basis of serial colony formations from single-plated hSSC, we do estimate ~50% of our hSSCs to represent bona fide stem cells. While the clones of a single hematopoietic stem cell can reconstitute entire blood and immune systems, it is reasonable to believe that the ability of a bone-forming stem cell to contribute to complete skeletal elements is much more limited. Consequently, most assays presented here monitor prospectively isolated hSSCs as a population versus at the single-cell level. Nonetheless, with the recent ascent of the hSSC field, as well as technological advances across scientific disciplines, we will observe the emergence of applications to study and trace human SSCs in vivo. This will allow to functionally define specific hSSC lineage subsets on the basis of alternative approaches taking advantage of clonal tracking in situ.

One additional caveat with our assays is the usage of immune deficient mice lacking an adaptive immune system for our human xenograft models, which potentially obscures the exact crosstalk between the immune system and human skeletal subset. However, it may be possible to address this limitation by also xenografting human hematopoietic stem cells and select immune subsets[36,37]. Other xenograft systems that we present, such as renal subcapsular and subcutaneous transplants, are heterotopic transplantation locations, and while they facilitate evaluation of the intrinsic skeletogenic activities of purified SSCs, skeletogenic cells transplanted in this location, are not subjected to the normal mechanical stress imposed on bones of the appendicular and axial skeleton. This may block out the role of mechanotransducive cues that may be needed for optimal hSSC activity. This limitation could potentially be addressed by utilizing novel types of biomechanically responsive

scaffolds, although we and others observe efficient bone and cartilage formation when hSSC are transplanted in Matrigel. However, Matrigel may also contain ill-defined growth factors that could alter the behavior of transplanted hSSC. To test the activity of growth factors that may stimulate hSSC differentiation in vivo, we suggest using Growth Factor Reduced Matrigel from Corning that could be further supplemented with defined factors.

The role of specific growth factors is also an important consideration in modifying and optimizing the in vitro assays that we present here. While many researchers utilize fetal bovine serum (FBS) for cell culture of skeletal progenitor cells, we have found that it is less suitable for hSSC culture as it seems to prevent differentiation. In substitute, we utilize human platelet lysate (HPL). However, we have also observed batch-dependent variability and it may be prudent to freeze multiple aliquots of a defined batch for long-term studies. Batch-to-batch variability is evident in the collagenase used for enzymatic digestion, and we recommend researchers to perform side-by-side comparisons of vendor lots to identify a suitable enzyme batch that achieves reproducible conditions across studies. It is also important to regularly test the pH of the collagenase solution as the pH should be ~6–7 for best enzymatic activity[38].

Other limitations include the reliance on technique-sensitive procedures such as FACS-based cell sorting, which some researchers may not have access to or be trained to conduct. Such technique-sensitive procedures can introduce user variability and bias. We have observed FACS profiles shifting depending on cytometer used and compensation setups applied. Despite those variations, prospectively isolated phenotypic hSSCs have been determined to display the same key stem cell characteristics across tissue sources[32].

A final potential limitation is the variability and accessibility of clinical specimens. We observe that some favored bone tissue sources, such as bone marrow reamings, yield very low numbers of hSSCs (Extended Data Fig. 2). While anatomical localization of hSSCs seems to play a role, we also suspect that tissue isolation techniques such as bone marrow reamings or aspirates generate heat that negatively impacts hSSC viability. Therefore, it is necessary to conduct appropriate controls to evaluate tissue viability before beginning the isolation procedures. Furthermore, obtaining clinical specimens for hSSC isolation may be difficult for researchers at institutions that are not directly engaged with an orthopedic surgery department. One solution is to find nearby collaborators at institutions that can help with access to such specimens. Finally, working with aborted fetal specimens, in cases where local laws do not prohibit it, should strictly follow rules set by ethical review boards.

Experimental design

Tissue dissociation (Steps 1–3)—Isolating skeletal stem cells from a variety of human tissues requires researchers to modify dissociation protocols depending on the type of skeletal source tissue. The goal of the mechanical dissociation is to break down the tissue into small enough pieces to maximize exposed surface area and allow for the collagenase buffer to effectively digest the remaining bonds (Fig. 1, Step 1A–C). Patient samples taken from fracture callus may have hard cortical bone, spongy trabecular bone or hardly any callus formation. The hard cortical bone may require bone scissors to break apart. However,

owing to this challenge and the general absence of hSSCs from cortical bone, any softer callus tissues obtained from the surgery are often more desirable.

Dissection scissors and razor blades are useful for the cartilaginous or softer tissues found in fetal bones and adult femoral head bone marrow (Fig. 2a, Step 1). The ideal consistency of the sample before incubation in collagenase resembles finely minced garlic and individual fragments should be under 100 μm in diameter. The key to effective dissociation is the collagenase digestion buffer, which utilizes type II collagenase to enzymatically break down cartilage matrix, HEPES buffer to maintain the pH, DNase to prevent cell clumping, $CaCl_2$ and bovine serum albumin (BSA) for enzyme stability, Pluronics (Poloxamer 199) to protect the cells from shearing stress and the nutrient-rich base media, Medium 199 (ref. [39]). Depending on blood presence in the sample, ammonium–chloride–potassium (ACK) lysis buffer is used, and preferred over gradient-based separation to deplete the red blood cells allowing for greater SSC yield (Steps 2–3).

Flow cytometry (Steps 4–22)—The unique surface-marker combinations present on the SSC and its downstream hierarchy enables analysis and isolation through flow cytometry (Fig. 2b,c and Extended Data Fig. 1, Steps 4–22). A six-color panel allows for a gating strategy to reliably isolate skeletal-lineage cells from multiple sources. Our gating strategy includes an initial forward versus side scatter to identify cells of interest based on size and granularity. A forward width versus area is then applied to capture all single cells. We then take all nonhematopoietic ($CD45^-CD235a^-$) and nonendothelial ($TIE2^-CD31^-$) populations and find hSSCs and their downstream populations through varying levels of PDPN, CD146, CD164 and CD73 expression. For example, to isolate hSSCs, we take the $PDPN^+CD146^-$ population and further gate the cells that are $CD73^+CD164^+$. Due to the variability in technique and potential differences in freshness of tissue, we recommend using fluorescence minus one (FMO) controls to guide your gating strategy for each FACS machine used and each sample source being analyzed to ensure the correct populations are gated (Extended Data Figs. 1 and 3, Step 21). Cell pellets will be incubated with rat immunoglobulin G to block endogenous Fc-γ receptors and reduce background during staining.

Human xenograft microfracture model—To utilize the benefits inherent in in vivo assays, we have developed a human xenograft mouse model (Fig. 3, Step 23A and Box 1). This allows for the study of the human tissue and cells in a more natural niche environment than can be achieved in vitro. This protocol describes the use of fetal phalanges as ideal xenograft tissues. After removing the skin and muscle, the fetal bone is placed subcutaneously on the dorsum of an NOD scid gamma (NSG) pup. The xenograft should be allowed to grow for at least 6 weeks to ensure maximal vascularization and engraftment (Fig. 3). To stimulate the resident hSSCs, microfracture surgery can be employed. Consequent hSSC expansion peaks within 1–2 weeks post-microfracture. The hSSCs can then be analyzed or isolated using our FACS protocol (Fig. 3b). Anti-human nuclear antigen antibody can be used later to distinguish donor from recipient contribution in explanted, sectioned histologically prepared specimens (Fig. 3e).

FACS-isolated patient hSSCs can also be transplanted into the microfracture site independently or in combination with recombinant factors to test their skeletal regeneration

capacity, as well as intramedullary inside the xenograft specimen. For easier hSSC tracking, the cells can efficiently be labeled with a fluorescence protein by lentiviral transduction (Box 2 and Fig. 3f). Overall, this xenograft microfracture technique is very important for assessing the response of activated hSSCs and their contribution to skeletal tissue upon injury. Moreover, we have also found that activated hSSCs can be skewed to cartilage regeneration through the guidance of extrinsic growth factors, and, thus, is a key utility for future clinical applications of understanding hSSC-mediated processes in situ.

Subcapsular renal transplantation (Step 23A)—The abundant blood supply of the renal cortex provides adequate nutrient supply for all kinds of transplants to rapidly engraft and expand under the renal capsule. However, successfully inserting the graft in the right location under the renal capsule within a short time requires accurate operation and knowledge of renal anatomy. In this paper, we provide a detailed protocol to ensure successful engraftment of transplants for isolated hSSCs and for fetal bone tissue (Fig. 4a–d, Step 23A).

The use of immunodeficient NSG mice as the host provides a hospitable, highly immunodeficient environment for engrafting human tissue in mice, which will minimize the possibility that distinct populations will be selected against or rejected due to human/mouse immunogenic differences. For cell transplants, it takes at least 2 weeks for transplanted cells to grow into tissue grafts under the kidney capsule; for whole bone transplants, it takes ~2 months before surgical excision and analysis of the graft. It is of importance to mention that the duration of graft to grow under renal capsule will dictate the skeletal output for examination. Specifically, it takes 3–4 weeks for endochondral formation to occur and for bone to be observed. If transient cartilage is the outcome, grafts should be excised sooner between 1 and 4 weeks post-surgery. Matrigel is used to encapsulate the cells before transplantation into renal capsule. Adult SSCs should be mixed with BioOss to enhance bone formation in vivo due to its physical and chemical similarity to mineralized matrix of human bone, which is necessary to induce bone formation (Fig. 4d).

After graft formation, the fate of transplanted hSSC and skeletal lineages can be assessed by Movat pentachrome, immunohistochemistry or redissociation and FACS analysis following the protocol described in paper. Optionally, reisolated graft cells can be used for retransplantation to probe in vivo stemness criteria such as long-term hSSC potential. However, additional care must be taken to optimize cell yield and eliminate cross-reactivity between mouse and human antigens by selecting the appropriate antibodies. The latter can be resolved by, for example, lentivirally transducing prospectively isolated hSSCs with green fluorescent protein (GFP) before transplantation (Box 2).

In vitro differentiation assays (Step 23B–D)—After isolation, hSSCs may be analyzed for their clonogenicity, osteogenic potential and chondrogenic potential in vitro (Fig. 4f, Step 23B–D). The CFU-F assay utilizes crystal violet to visualize the number of colonies formed from a set number of plated skeletal lineage cells (Step 23B). Dissociating and resorting picked colonies can be used to probe stem cell-like serial plating capacity. The osteogenic assay relies on osteogenic factors to differentiate the hSSC toward osteoblasts. After differentiation, Alizarin red S stains the calcium deposits produced as a measure

of bone forming potential (Step 23C). The chondrogenic assay is a micromass method that relies on differentiation factors to produce cartilage in vitro (Step 23D). Alcian blue stains for proteoglycans as a measure of the cell's cartilage-forming potential. These in vitro assays should be used as additional methods in lieu of the more definitive in vivo transplantation methods described above for testing hSSC stemness and differentiation capacity. While we have observed minor differences during expansion and differentiation assays of hSSCs at hypoxic and normoxic conditions (unpublished), cells tend to perform better at 2% oxygen.

ScRNAseq (Step 23E)—hSSCs can be directly index-sorted into 96-well lysis plates for downstream scRNAseq analysis (Fig. 5, Step 23E) based on a slightly modified SmartSeq2 protocol by Picelli et al.[40]. ScRNAseq enables the investigation of single hSSC full-length transcriptomes using high-throughput technology, e.g., to explore diversity, detect novel highly expressed markers that could be used for further separation or investigate the role of alternative splicing. This technique can also be implemented to test hSSC heterogeneity within various bone types, ages, genders, disease, etc. Additionally, this strategy can be utilized to find new markers for hSSC identification and visualization in situ. The analysis of scRNAseq requires computational skills using Seurat and R-studio.

Lastly, SmartSeq2 is not the only scRNAseq platform that can be used for hSSC analysis; other methodologies include 10X Genomics droplet-based and other microfluidic scRNAseq assays[41]. However, since hSSCs comprise a rare cell population and droplet-based approaches such as 10X Genomics need high cellular input, plate-based approaches are preferred as in most cases have dramatically higher gene recovery rates than comparable droplet-based approaches[42]. Despite the lower throughput with SmartSeq2, this allows full-length RNA sequencing, generating higher genes per cell coverage and facilitating additional interrogations into alternative splicing. To increase workflow of cellular processing with this approach, flow cytometric sorters equipped with a sorting option into 384-well plates could be used alternatively (at half the volumes presented for the 96-well plate format here).

Materials

Biological materials

- Human femoral heads (can be obtained from hip replacement surgeries and should be processed as soon as possible) ▲**CRITICAL** Samples should be processed the same day to maximize viable cell yield. **!CAUTION** Appropriate national laws and institutional regulatory board guidelines must be followed. Adult femoral heads were obtained by Stanford Hospital in accordance with guidelines set by the institutional review board (IRB-35711). On this IRB, informed consent was not required as samples were considered biological waste. No restrictions were made regarding the race, gender or age of the specimen's donor. Following excision, all femoral head specimens were placed on ice, and skeletal stem and progenitor cells were isolated from the bone marrow cavity and articular cartilage, as described below.

- Human fracture callus (can be obtained from open reduction internal fixation orthopedic surgeries—during those surgeries the fracture sites are debrided of newly formed callus tissues rich in hSSCs that are generally considered medical waste ▲**CRITICAL** Samples should be processed as soon as possible. If necessary, samples can be stored at 4 °C in cell suspension buffer overnight at a risk of reduced cell viability. **!CAUTION** Appropriate national laws and institutional regulatory board guidelines must be followed. Fracture calluses were obtained by Stanford Hospital in accordance with guidelines set by the IRB (IRB-35711). On this IRB, informed consent was not required as samples were considered biological waste. No restrictions were made regarding the race, gender or age of the specimen's donor.
- Human fetal bone samples (Stemexpress, shipped overnight; our samples ranged in age from 10 to 20 weeks of gestation with no restrictions on race or gender) **!CAUTION** Appropriate national laws and institutional regulatory board guidelines must be followed. Fetal sample procurement and handling was in accordance with the guidelines set by the IRB, Stanford University (IRB-35711).
- Human monocyte-derived iPSCs line SCVI 113; RRID: CVCL_A8QC (ours was a kind gift from the laboratory of Joseph C. Wu (joewu@stanford.edu); the donor was a 51-year-old man) **!CAUTION** Any experimental protocol using hiPSCs must comply with national and regional laws and institutional ethical guidelines and regulations. **!CAUTION** Cell lines should be regularly checked to ensure they are authentic and are not infected with mycoplasma.
- Mice: NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (commonly referred to as NSG mice) (JAX, cat. no. 005557) male or female mice aged 3–5 d postnatal for human xenograft model and 2–3-month-old female mice (renal transplant) **!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 and received approval from the IRB.

Reagents

!CAUTION Refer to the safety information provided by the vendors and take necessary precautions when handling reagents and equipment. Use proper personal protective equipment at biosafety level 2 for tissue culture work.

- 100% oxygen tank (Gas Cylinder Source, cat. no. 20STEEL-540)
- Accudrop Beads (BD Biosciences, cat. no. 345249)
- Agencourt AMPure XP beads (Beckman Coulter, cat. no. A63882)
- Albumin from bovine serum (Sigma-Aldrich, cat. no. A4503–50G)
- Alcian blue 8GX (Sigma-Aldrich, cat. no. A5268)
- Alizarin red S (Sigma-Aldrich, cat. no. A5533)

- ACK lysis buffer (Gibco, cat. no. A10492–01)
- β-Glycerophosphate disodium salt hydrate (Sigma-Aldrich, cat. no. G9422)
- Calcium chloride (Sigma-Aldrich, cat. no. C5670)
- Ciprofloxacin (Sigma-Aldrich, cat. no. 17850)
- Collagenase type II from *Clostridium histolyticum* (Sigma-Aldrich, cat. no. C6885) **!CAUTION** Collagenase type II is considered a hazardous substance and should be handled inside a chemical fume hood.
- Crystal violet (Sigma-Aldrich, cat. no. C0775)
- 4,6-Diamidino-2-phenylindole (DAPI) (10 mg) (BioLegend, cat. no. 422801) ▲**CRITICAL** Since DAPI is photosensitive, be sure to minimize exposure to light sources.
- Deoxyribonuclease 1, Ribonuclease & Protease Free, 500 U (Worthington, cat. no. LS006344)
- Dexamethasone (MP Biomedicals, cat. no. 02194561)
- Dulbecco's modified Eagle medium (DMEM)/F-12 medium (Gibco, cat. no. 11320–033)
- DMEM high glucose medium (Gibco, cat. no. 10569–010)
- Opti-MEMI Reduced-Serum Medium (Gibco, cat. no. 31985070)
- Lipofectamine 3000 Transfection Reagent (Invitrogen, cat. no. L3000001)
- Polybrene Infection/Transfection Reagent (Sigma, cat. no. TR-1003-G
- Ethylenediaminetetraacetic acid (EDTA), 5 mM (Gibco, cat. no. 15575–020)
- ERCC (External RNA Controls Consortium) ExFold RNA Spike-In Mixes (Life Technologies, cat. no. 4456739)
- Essential 8 medium (Gibco, cat. no. A1517001)
- FBS (Gibco, cat. no. 16000–069)
- Geltrex (Gibco, cat. no.A1569601)
- Heparin (Novaplus, cat. no. 63323–540-57)
- HEPES buffer (Gibco, cat. no. 15–630-080)
- High Sensitivity DNA analysis kit (Agilent Technologies, cat. no. 5067–1504)
- Histopaque-1119 (Sigma-Aldrich, cat. no. 11191)
- Human platelet lysate (Supply Clinic, cat. no. 169–0ZZ)
- Helistat Hemostatic Sponge (StemCell Technologies, cat. no. 06962)
- ISPCR (in situ polymerase chain reaction) primers (5′-AAGCAGTGGTATCAACGCAGAGT-3′) (IDT, special order)

- Indomethacin (Sigma-Aldrich, cat. no. I7378)
- Isoflurane (Fluriso, VetOne, cat. no. 502017, NDC: 13985–528-60) **!CAUTION** Isoflurane is an anesthetic gas. Anesthetic machines should be equipped with a scavenging system for removal of waste isoflurane and should be operated in a well-ventilated room. Store isoflurane in a locked cabinet.
- KAPA HiFi hotStart ReadyMix (Kapa Biosystems, cat. no. KK2602)
- L-Ascorbic acid 2-phosphate (Sigma-Aldrich, cat. no. A8960)
- Locked template-switching oligonucleotide (TSO) (5′-AAGCAGTGGTATCAACGCAGAGTACATrGrG +G-3′) (Exiqon, special order)
- Growth Factor Reduced Matrigel membrane matrix (Corning; cat. no. 354230)
- Matrigel membrane matrix (Corning; cat. no. 356234)
- Medium 199 (Sigma-Aldrich, cat. no. M4530)
- MEMα (minimum essential medium alpha) medium (Gibco, cat. no. 32561–037)
- $MgCl_2$ anhydrous (Sigma-Aldrich, cat. no. M8266)
- Mouse anti-human CD31 (PECAM-1), clone: WM-59, Biotin (100 μg) (Invitrogen, cat. no. 13–0319-82; RRID: AB_466423)
- Mouse anti-human CD45, clone: H130, Pacific Blue (200 μg) (BioLegend, cat. no. 304029; RRID: AB_2174123) ▲**CRITICAL** As Pacific Blue is photosensitive, be sure to minimize exposure to light sources.
- Mouse anti-human CD73, clone: AD2, fluorescein isothiocyanate (FITC) (200 μg) (BioLegend, cat. no. 344016; RRID: AB_2561809) ▲**CRITICAL** As FITC is photosensitive, be sure to minimize exposure to light sources.
- Mouse anti-human CD146 (MUC18/Mel-CAM), clone: SHM-57, phycoerythrin (PE)/cyanine7 (100 μg) (BioLegend, cat. no. 342010; AB_10644009) ▲**CRITICAL** As PE/cyanine7 is photosensitive, be sure to minimize exposure to light sources.
- Mouse anti-human CD164, clone: 67D2, PE (25 μg) (BioLegend, cat. no. 324808, RRID: AB_2072587) ▲**CRITICAL** As PE is photosensitive, be sure to minimize exposure to light sources.
- Mouse anti-human CD202b (Tie2/Tek), clone: 33.1, Biotin (100 μg) (BioLegend, cat. no. 334204; RRID: AB_1186046)
- Mouse anti-human CD235ab, clone: HIR2, Pacific Blue (100 μg) (BioLegend, cat. no. 306612; RRID: AB_2116241) ▲**CRITICAL** As Pacific Blue is photosensitive, be sure to minimize exposure to light sources.
- Mouse anti-human Podoplanin, clone: NZ-1.3, Allophycocyanin (APC) (125 μg) (Invitrogen, cat. no. 17–9381-42; RRID: AB_10801951) ▲**CRITICAL** As APC is photosensitive, be sure to minimize exposure to light sources.

- Nextera XT DNA Library Prep kit (Illumina, cat. no. FC-131–1096)
- NextSeq 500** (Illumina)
- Oligo dT30 VN (5′-AAGCAGTGGTATCAACGCAGAGTACT30VN-3′) (IDT special order)
- OneComp eBeads Compensation Beads (Invitrogen, cat. no. 01–1111-42)
- Paraformaldehyde (PFA) Aqueous Solution, EM Grade, Ampoule 10 ml (Electron Microscopy Sciences, cat. no. 15710) **!CAUTION** PFA is toxic and must always be handled with care inside a chemical fume hood.
- Penicillin–streptomycin (Gibco, cat. no. 15140–122)
- Phosphate-buffered saline (PBS) (Gibco, cat. no. 10010–023)
- Poloxamer 188, 10% (Sigma-Aldrich, cat. no. P5556)
- Povidine–iodine solution, 10% (Major Pharmaceuticals, cat. no. B0165W97UA)
- Qubit dsDNA HS assay kits (Invitrogen, cat. no. Q33231)
- Recombinant human BMP2 (R&D, cat. no.355-BM-010)
- Recombinant human TGF-β 1 protein (R&D, cat. no. 240-B)
- Recombinant RNase inhibitor (Clonetech, cat. no. 2313B)
- SMARTScribe Reverse Transcriptase (Clonetech, cat. no. 639538)
- Streptavidin, APC-Alexa Fluor 750 (1 ml) (Invitrogen, cat. no. SA1027) ▲**CRITICAL** As APC-Alexa Fluor 750 is photosensitive, keep aliquots wrapped in foil to avoid light.
- Trypan blue solution, 0.4% (Gibco, cat. no. 15250–061)
- Trypsin–EDTA (0.05%), phenol red (Gibco, cat. no. 25300–054)
- TSO (Qiagen, special order)

Equpment

- 6–0 ethilon nylon suture (Ethilon, cat. no. 697H)
- Absorbent underpad (Fisherbrand, cat. no. 14–206-62)
- Cell culture-treated dish, 10 cm (Falcon, cat. no. 353003)
- Cell culture-treated plate, 6 well (Falcon, cat. no. 353046)
- Cell culture-treated plate, 48 well (Falcon, cat. no. 353078)
- Cell culture-treated plate, 96 well (Falcon, cat. no. 353072)
- Cell strainer, 100 μm (Falcon, cat. no. 352360)
- Conical centrifuge tubes (15 ml and 50 ml; Falcon, cat. no. 352096 and 352070)

- Culture-treated microplate (6 well, 48 well, 96 well; Falcon, cat. nos. 353046, 353078, 353072, respectively)
- Disposable 10 blade scalpel (Exelint, cat. no. 29550)
- Filter steriflip, 0.22 μm pore size (50 ml; MilliporeSigma, cat. no. SCGP00525)
- Filter stericup, 0.22 μm pore size (250 ml and 500 ml; MilliporeSigma, cat. no. S2GPU02RE and S2GPU05RE)
- Gloves (S–XL; Fisherbrand, cat. no. 19–130-1597B, C, D, E)
- Microcentrifuge tube, 1.5 ml (Corning, cat. no. 04120077)
- Petri dish, 10 cm (Falcon, cat. no. 351029)
- Pipette tips (1,000 μl, 200 μl, 20 μl; ThermoFisher, cat. nos. 2779HR, 2769HR, 2749HR, respectively)
- Razor blade (Excelta, cat. no. 176–1)
- Round-bottom polystyrene test tubes with 40 μm cell strainer snap cap (Falcon, cat. no. 352235)
- Trocar needle (SRA-Soldier, cat. no. TIPS#22×1.5)
- Beebee bone scissors (F.S.T., cat. no. 16044–10)
- Beckman model GS-6KR floor model centrifuge with rotor
- Bioanalyzer (Agilent, cat. no. G2939BA)
- Blunt forceps (F.S.T., cat. no. 11010–17)
- Certomat BS-1 incubation shaker (Sartorius, cat. no. 14–559-203)
- Cell incubator (Thermo, Model: HERAcell 150i, cat. no. 51026554)
- Dissection microscope (Leica, cat. no. S6D-PS)
- Dumont 55 forceps (F.S.T., cat. no. 11255–20)
- Electronic pipette controller (Drummond, cat. no. 4–000-101)
- Fine scissors-ceramacut (F.S.T, cat. no.14958–09)
- Flow Cytometer (BD, model FACS Aria II)
- FlowJo (https://www.flowjo.com/solutions/flowjo/downloads)
- Halsey micro needle holder (F.S.T, cat. no. 12500–12)
- Hemocytometer (Hausser Scientific, cat. no. 3200)
- Iris forceps (F.S.T., cat. no. 11064–07)
- Ophthalmic forceps (F.S.T., cat. no. 11255–20)
- Pipette (1,000 μl, 200 μl, 20 μl, 2 μl, Rainin, cat. nos. 17014382, 17014391, 17014392, 17014393, respectively)

- Razor (Fisherbrand, cat. no. 12640)
- Toothed forceps (F.S.T, cat. no. 11071–10)
- Trocard-wire troll 2 (Drummond Scientific, cat. no.5–000-2005)
- Qubit 2.0 Fluorometer (Invitrogen, cat. no. Q33226)
- Vaporizer Anesthesia Masks (Kent Scientific, cat. no. VetFlo-0801)
- Vaporizer for Isoflurane (Kent Scientific, cat. no. VetFlo-1231)
- Vaporizer Sliding Top Induction Chamber (Kent Scientific, cat. no. VetFlo-0530SM)
- VetFlo Two Channel Anesthesia Stand (Kent Scientific, cat. no. VetFlo-1215)

Reagent setup

Plate coating with gelatin—Add 0.1% (wt/vol) gelatin to cover culture plates, and incubate for 15 min. Aspirate the gelatin immediately before culturing the cells. ▲**CRITICAL** All cell culture plates should be coated with gelatin.

Cell suspension buffer—Prepare calcium- and magnesium-free PBS containing 2% FBS, 1 mg ml^{-1} Poloxamer 188, and 100 U ml^{-1} penicillin–streptomycin. Filter through a 0.22 μm pore size membrane and store at 4 °C for up to 2 weeks.

Digestion buffer—Prepare Medium 199 containing 2.2 mg ml^{-1} collagenase type II, 100 U ml^{-1} DNAse, 1 mg ml^{-1} Poloxamer 188, 100 μg ml^{-1} BSA, 20 mM HEPES buffer and 1 mM $CaCl_2$. Filter through a 0.22 μm pore size membrane and store at 4 °C for up to 1 week.

Culture medium—Prepare MEMα medium containing 10% HPL, 100 U ml^{-1} penicillin–streptomycin, ciprofloxacin and heparin. Filter through a 0.22 μm pore size membrane and store at 4 °C for up to 4 weeks.

Osteogenic differentiation medium—Prepare MEMα medium containing 10% HPL, 100 U ml^{-1} penicillin–streptomycin, 0.4 mM ascorbic acid, 10 μM β-glycerophosphate and 0.1 μM dexamethasone. Filter components through a 0.22 μm pore size membrane. The stocks of the ascorbic acid, β-glycerophosphate and dexamethasone may be prepared in advance and frozen at −20 °C, but osteogenic differentiation medium must be prepared fresh at each medium change.

Chondrogenic differentiation medium—Prepare DMEM high-glucose medium containing 10% HPL, 100 U ml^{-1} penicillin–streptomycin, 1 μM ascorbic acid, 10 ng ml^{-1} transforming growth factor β1 and 100 nM dexamethasone. Filter components through a 0.22 μm pore size membrane. The stocks of the ascorbic acid, transforming growth factor β1 and dexamethasone may be prepared in advance and frozen at −20 °C, but chondrogenic differentiation medium must be prepared fresh at each medium change.

hSSC differentiation medium from hiPSCs—Prepare MEM-alpha medium supplemented with 100 nM of dexamethasone, 10 nM β-glycerophosphate, 2.5 mM ascorbic acid 2-phosphate and 50 ng ml^{-1} of rhBMP2. Filter components through a 0.22 μm pore size membrane. The stocks of the ascorbic acid, β-glycerophosphate, dexamethasone and rhBMP2 may be prepared in advance and frozen at −20 °C, but chondrogenic differentiation medium must be prepared fresh at each medium change.

Lysis mixture for SmartSeq2 single-cell platform (per 96 well)—Combine 3.262 μl nuclease-free water, 0.1 μl RNAse inhibitor, 0.1 μl of ERCC previously diluted (1:600,000), 0.038 μl 10% Triton, 0.4 μl 25mM dNTP mix and 0.1 μl 100 μM oligo dT30VN. Prepare fresh on the day of sorting.

Reverse transcription mixture for SmartSeq2 single-cell platform (per 96 well)—Combine 1 μl Clonetech Smartscribe (100 U μl^{-1}), 0.25 μl RNAse inhibitor (40 U μl^{-1}), 2 μl First Strand Buffer (5×), 0.5 μl DTT (100 mM), 2 μl betaine (5 M), 0.06 μl $MgCl_2$ (1 M), 0.1 μl TSO (100 μM) and 0.0 9 μl nuclease-free water. Prepare fresh at time of use.

Polymerase chain reaction preamplification mixture for SmartSeq2 single-cell platform (per 96 well)—Combine 12.5 μl KAPA Hifi HotStart ReadyMix (2×), 0.25 μl ISPCR Primers (10 μM) and 2.25 μl nuclease-free water. Prepare fresh at time of use.

Equipment setup

Flow cytometer—Ensure the flow cytometer is equipped with the necessary filters and lasers 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)
APC-AF750	640	780/60 BP
PE	561	582/15 BP
FitC	488	525/50 BP
APC	640	670/30 BP
PE-Cy7	561	780/60 BP
Pacific Blue	405	450/50 BP

BP, band pass.

Procedure

Tissue dissociation ● Timing 1.5 h to 3 weeks

!CAUTION When working with human samples, it is important to follow all standard blood-borne precautions including wearing gloves, a laboratory coat, goggles and any other necessary personal protective equipment to minimize risk.

1. Purified hSSCs, hOPs or hCPs can be obtained from several sources through the utilization of FACS to dissociate cells from the tissue of interest in the case of fracture calluses (A), adult femoral heads (B) and fetal bones (C), or differentiate from iPSCs (D) before antibody staining and FACS analysis.

(A) Fracture callus ● Timing 1.5 h

i. Prepare a clean area and cover the bench with an absorbent underpad.

ii. Wash the callus tissue with cell suspension buffer to remove excess blood.

iii. Dissect away any large black/dark-red blood clots if present.

▲**CRITICAL STEP** Removal of blood clots is critical to generate cell suspensions with high viability.

iv. Place the fracture callus tissue in a 10 cm Petri dish.

v. Add ~500 μl digestion buffer.

vi. Mince using a razor blade. Due to the heterogeneous nature of human calluses, different techniques are useful with different callus types:

- Periosteal and soft tissue attached to cortical bone can be scraped with the razor blade and the slightly mineralized portions removed with bone scissors. Any bone too tough to process using bone scissors may be discarded
- Trabecular bone can be cut with surgical scissors and minced with the razor blade
- Soft, fibrous tissues can be cut with dissection scissors to minimize splashing

vii. Once fully minced, transfer the sample to a 50 ml conical tube and bring the volume up to ~10 ml with fresh digestion buffer. Seal the tube with parafilm to prevent any leakage.

viii. Incubate sample at 37 °C at an angle with a shaking speed of 210 rpm for 1 h.

▲**CRITICAL STEP** Allowing the sample to shake at an angle promotes digestion of the whole sample, increasing cell yield.

ix. After incubation, briefly vortex the sample and fill to 50 ml with cold cell suspension buffer. ▲**CRITICAL STEP** We recommend quenching the digest with at least twice the volume of the cell suspension buffer.

? TROUBLESHOOTING

x. Filter the digest through a 100 μm filter into a clean 50 ml conical tube.

xi. Centrifuge the diluted digest at 200*g* for 5 min at 4 °C. Discard supernatant.

xii. As fracture callus-derived cell pellets typically contain blood, proceed to Step 2 to perform ACK red blood cell lysis.

(B) Adult femoral head or bone marrow aspirates ● Timing 2–3 h

i. Prepare a clean area and cover the bench with an absorbent underpad.

ii. Secure the femoral head in a clamp to prevent slipping.

iii. Using a sharp tool, such as closed dissection scissors, loosen the bone marrow.

iv. Scoop out trabecular bone rich marrow amounting roughly to size of a fingernail (~0.125 cm^3) and collect in a Petri dish on ice.

v. Add 2 ml of digestion buffer to the Petri dish and chop up the bone marrow with a razor blade. While chopping, the Petri dish should be kept on ice.

vi. Collect chopped bone marrow in a 50 ml conical tube. Add digestion buffer up to 15 ml. Seal with parafilm to prevent leakage.

vii. Incubate the sample at 37 °C at an angle with a shaking speed of 210 rpm for 1 h.

▲**CRITICAL STEP** Allowing the sample to shake at an angle promotes digestion of the whole sample, increasing cell number.

viii. After incubation, briefly vortex the sample and fill to 50 ml with cold cell suspension buffer. Let the bone fragments settle for 2 min.

▲**CRITICAL STEP** We recommend quenching the digest with at least twice the volume of cell suspension buffer.

? TROUBLESHOOTING

ix. Without disturbing the bone fragments at the bottom, pipette out the cell suspension and filter the digest through 100 μm filters into a clean 50 ml conical tube.

x. Centrifuge the diluted digest at 200*g* for 5 min at 4 °C.

xi. Proceed to Step 2 to begin red blood cell lysis.

(C) Fetal bone ● Timing 1.5 h—▲CRITICAL Dissection of fetal bones is dependent upon the body part received and varies to accommodate this. The following general technique can be followed in most cases.

i. Prepare a clean area and cover the bench with an absorbent underpad.

ii. Use forceps to gently remove most of the surrounding connective tissue.

iii. If a tough tissue is encountered, use dissection scissors.

iv. After the bone is removed, gently clean it by placing it in a paper towel and using a gentle rolling motion.

▲**CRITICAL STEP** This should be done briefly. Drying of the tissue can lead to cell death.

■**PAUSE POINT** Dissected, cleaned bones can be left in cell suspension buffer on ice until ready to proceed to either dissociation or transplantation in Box 1.

v. Once all the desired bones have been dissected and cleaned, place the fetal bone in a 10 cm Petri dish on ice.

vi. Add ~500 μl of digestion buffer and begin to mince using a razor blade.

vii. Once fully minced, transfer the sample to a 50 ml conical tube and bring the volume of the tube up to ~10 ml with fresh digestion buffer. Seal the tube with parafilm to prevent any leakage.

viii. Incubate sample at 37 °C at an angle with a shaking speed of 210 rpm for 1 h.

▲**CRITICAL STEP** Allowing the sample to shake at an angle promotes digestion of the whole sample, increasing cell number.

ix. After incubation, briefly vortex the sample and fill to 50 ml with cold cell suspension buffer. ▲**CRITICAL STEP** We recommend quenching the digest with at least twice the volume of cell suspension buffer.

? TROUBLESHOOTING

x. Filter the digest through a 100 μm strainer into a clean 50 ml conical tube.

xi. Centrifuge the diluted digest at 200*g* for 5 min at 4 °C.

xii. In cases where the sample appears bloody, proceed to Step 2 to perform ACK lysis. Otherwise skip to Step 4 to begin antibody staining.

(D) IPSC differentiation ● Timing 2–3 weeks

i. Maintain iPSCs in Essential 8 Medium, feeding every day. Begin induction when iPSCs are at 80% confluency.

ii. Add hiPSC differentiation medium to cells and feed every other day.

▲**CRITICAL STEP** To maintain cell viability, cultures must be fed every other day.

iii. After 2 weeks of differentiation, aspirate the culture medium and wash the plate with enough calcium- and magnesium-free PBS to fully cover the layer of cells.

iv. Add enough 0.05 mM EDTA to fully cover the layer of cells and incubate for 4–5 min. Carefully aspirate off the EDTA without disrupting the cells.

▲**CRITICAL STEP** Use a microscope to ensure cells are not lifting from the plate before aspirating the EDTA.

v. Add 10 ml of cell suspension buffer to the plate, pipetting up and down to dislodge the cells.

vi. Transfer to a 15 ml conical tube and centrifuge at 200*g* for 5 min at 4 °C. (vii) Proceed to Step 4 to begin antibody staining.

Red blood cell lysis ● Timing 10 min

2. After centrifuging, decant the supernatant and add 2 ml ACK lysis buffer to the pellet. Using a P1000 gently pipette up and down to ensure the pellet is fully

resuspended. Incubate for 3 min at room temperature (24–26 °C). More ACK lysis buffer may be needed if the volume of red blood cells exceeds 50% of the pellet.

3. After 3 min, quench the ACK lysis buffer with at least 15 ml of cell suspension buffer. Quench with more if additional ACK lysis buffer was used. Centrifuge the sample at 200*g* for 5 min at 4 °C. Proceed to antibody staining.

   ▲**CRITICAL STEP** We recommend quenching the ACK lysis buffer with a ratio of at least 3:1 cell suspension buffer.

? TROUBLESHOOTING

Antibody staining ● Timing 1.5 h

4. After centrifuging, aspirate the supernatant and resuspend in 150 μl of cold cell suspension buffer.
5. Transfer 100 μl of sample into a round bottom polypropylene microcentrifuge tube for the experimental fully stained sample, and 5 μl each into seven separate 1.5 ml microcentrifuge tubes for the six FMO controls and the unstained control.
6. Stain the six FMO controls and experimental sample based on the antibody concentrations provided in Table 1 in the dark, for 30 min, on ice.
7. Wash the primary antibody off by adding 1 ml of cell suspension buffer to the sample.
8. Centrifuge the tube at 200*g* for 5 min at 4 °C.
9. Carefully aspirate away supernatant and resuspend the cells in 100 μl of cell suspension buffer.
10. Add the secondary antibody at a concentration provided in Table 1 to the sample and incubate in the dark for 30 min, on ice.
11. Wash off the secondary antibody by adding 1 ml of cell suspension buffer to the sample.
12. Centrifuge the tube at 200*g* for 5 min at 4 °C.
13. Remove supernatant. Resuspend in 100 μl of cell suspension buffer.
14. Use a test tube with a 40 μm cell strainer cap to filter the suspension, and rinse the cap with 100 μl of cell suspension buffer immediately before running flow cytometry

    ▲**CRITICAL STEP** Filtering immediately before running flow cytometry will help prevent clogging of the machine.

Flow cytometry ● Timing 2 h

15. Use a 100 μm nozzle and steady the stream.

16. Perform fluorescence compensation to correct for spectral overlap. 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.

17. Using AccuDrop Beads, calculate drop delay to ensure that the correct droplet will be selected for sorting.

18. Align the stream to target the center of the collection tube by adjusting the plate voltages.

19. Turn on the chiller and the aerosol management system to maintain a sterile 4 °C environment for sorting.

    !CAUTION Patient-derived cells may contain pathogens and correct use of the aerosol management system can protect the user from unintentional exposure. Flow cytometry should also be performed in a biosafety level 2-certified laboratory.

20. Add 1μg ml^{-1} of the live–dead dye, DAPI, to the samples.

21. Run FMO samples first to establish the gating strategy for sorting. Generate the following panels for optimal results:

    - SSC-A versus FSC-A to capture all cells
    - FSC-W versus FSC-A to capture singlets
    - TIE2/CD31 versus CD45/CD235a/DAPI to capture all live (DAPI-), nonhematopoietic (CD45, CD235a), CD31-, TIE2- cells
    - PDPN versus CD146
    - CD73 versus CD146

22. Sort your desired cell population(s) into 200 μl of chilled cell suspension buffer in a collection tube on ‘purity’ setting for downstream assays, Alternatively, for scRNAseq, cells can be sorted on single-cell index mode into a 96-well plate containing 4 μl lysis buffer before spinning down and freezing the plate immediately at −80 °C. Note: cells may be double-sorted on the ‘yield’ setting first followed by the ‘purity’ setting for higher purity.

Downstream assays

23. At this point in the protocol, proceed with the following downstream functional assays: (A) in vivo renal capsule transplantation, (B) in vitro colony formation, (C) in vitro osteogenesis, (D) in vitro chondrogenesis or (E) single-cell sequencing assays described below. Additionally, freshly sorted hSSCs can be labeled with fluorophore using lentiviral transduction for subsequent labeling before downstream assays (Box 2), e.g., for their identification upon transplantation into a murine host and/or reisolation from the xenograft setting for secondary transplantation assays.

(A) In vivo renal subcapsular transplant ● Timing 1 h—!CAUTION All experiments involving mice should be performed in accordance with relevant guidelines and institutional regulations.

▲**CRITICAL** For transplanting cells, at Step 22, sort 20,000 fetal hSSCs into 200 µl of chilled FACS buffer in a 1.5 ml Eppendorf tube.

For cell transplants:

- Centrifuge the cells at 200*g* for 5 min at 4 °C
- Aspirate the supernatant and resuspend the cells in 2 µl Matrigel per graft
- Load the Matrigel cell suspension into a trocar needle and keep it on ice until the kidney is ready for the transplant

For whole bone transplants:

- Dissect out the fetal bone tissue for transplantation and keep it on ice in FACS buffer until ready for transplantation. Ensure it is clean and of a suitable size for transplantation
- Keep tissue moisturized with FACS buffer and leave it on ice until transplantation

  ▲**CRITICAL STEP** Allowing the tissue to dry out can lead to degradation and cell death.

i. Set up an isoflurane gas system with both an induction chamber and a nose cone, with the nose cone in a position that would allow visualization of the mouse abdomen through the dissection microscope. Begin with 3 l min^{-1} of 3% (vol/vol) isoflurane in 100% (vol/vol) oxygen flowing only into the induction chamber.

ii. Place the 8- to 12-week-old NSG mouse in the induction chamber until the breathing pattern has slowed. Check for the absence of the righting reflex or toe pinch response.

▲**CRITICAL STEP** An NSG mouse should be used as the recipient to avoid immune rejection.

iii. Adjust the position of the mouse and nose cone to ensure both consistent gas flow through the nose cone to the mouse and visualization of the upper abdominal area through the microscope during the surgery.

iv. Use a razor to shave the hair off the lateral abdomen on the surgical side.

v. Palpate the kidney area below the costovertebral angle until you feel the kidney. Mark the skin with toothed forceps where the kidney is pressing most firmly against the skin surface (Fig. 4b1).

▲**CRITICAL STEP** Cutting into the skin at the correct area minimizes error down the line.

vi. Sterilize the shaved area with 70% ethanol and povidone–iodine solution.

vii. Drape the mouse with sterile drapes, leaving the surgical site exposed (Fig. 4b2).

viii. Make an incision at the marked skin using scissors and forceps.

ix. Expose the abdominal wall by blunt dissection.

x. Confirm the kidney's physiological location (Fig. 4b3).

▲**CRITICAL STEP** The kidney can be identified by its deep red color and bean-like shape. Correct identification of the physiological location of the kidney is essential.

xi. Lift up the abdominal wall above the kidney with ophthalmic forceps, and make a <1 cm incision along the line of its fibers.

xii. Gently squeeze the kidney outside the abdominal cavity through this incision. A smaller cut on the abdominal wall helps to hold the kidney in position during transplantation (Fig. 4b4).

xiii. Keep the surface of kidney moisture during surgery by drop added sterilized PBS or saline.

▲**CRITICAL STEP** Do not allow the kidney to dry out.

xiv. For cell transplants, move the trocar to room temperature shortly before transplantation to solidify the Matrigel.

xv. Use the tip of a 16-gauge needle to gently make a shallow cut of ~1 mm into the renal capsule on the side of the kidney.

xvi. Make a blunt beveled edge at a 45° angle on a 20 μl pipette tip (Fig. 4b5). Note: may be prepared in advance.

xvii. Gently separate the capsule from the renal parenchyma by horizontally inserting the prewetted tip through the incision made by the 16-gauge needle (Fig. 4b5). Be sure to create a tunnel large enough for the transplant by gently wiggling the pipette tip until a sufficient space is created (Fig. 4b7).

xviii. Insert the graft (either the trocar with cells or a fetal tissue specimen) under the renal capsule and push the graft to the bottom of the space created (Fig. 4b8).

xix. Place a piece of sponge after the graft under the capsule to keep the graft in position.

xx. Gently pull the abdominal wall to let the kidney drop back into the abdominal cavity.

xxi. Close the abdominal wall with simple interrupted sutures (Fig. 4b9).

xxii. Close the skin with 6–0 everting sutures or staples (Fig. 4b10).

xxiii. For cell transplants, wait 2–4 weeks for a graft to grow under the kidney capsule before performing analysis (Fig. 4b). For fetal bone tissue transplants, wait 2 months before surgical excision and analysis of graft.

? TROUBLESHOOTING

(B) In vitro colony formation ● Timing 2 weeks—▲CRITICAL For in vitro colony formation, at Step 22, sort 200 hSSCs, hOPS or hCPs directly into a six-well plate with 2 ml of complete MEMα medium per well.

i. Incubate the plate of sorted cells at 37 °C at low O_2 (2% (vol/vol) atmospheric oxygen, 7.5% (vol/vol) CO_2) conditions.

ii. Replace the medium with fresh medium every 2–3 d.

iii. After 14 d of incubation, dilute 16% PFA to 4% with PBS, remove the medium and fix the cells with 4% PFA for 20 min at room temperature.

iv. Prepare fresh 0.5% crystal violet in methanol carefully.

v. After 20 min, remove the PFA and add 1.5 ml 0.5% crystal violet to each well.

vi. Incubate at room temperature for 20 min.

vii. After 20 min, remove the crystal violet and wash each well with water three times.

viii. Allow to dry for imaging and subsequent quantification through counting the stained colonies.

(C) In vitro osteogenesis ● Timing 2 weeks for expansion, then 2 weeks for differentiation—▲CRITICAL To ensure that after expansion there are sufficient numbers of hSSCs, hOPs or hCPs for differentiation assays, at Step 22, sort at least 500 cells into a microcentrifuge tube.

i. Transfer the entire contents of the microcentrifuge tube of sorted cell into a 10 cm tissue culture plate for expansion with 10 ml of culture medium. Swirl the plate gently to evenly distribute the cells.

ii. Incubate the cells at 37 °C at low O_2 conditions for 2 weeks with a medium change every 2–3 d.

iii. After expansion lift cells with 1 ml 0.05% trypsin and quench with two times the volume of complete MEMα medium.

iv. Spin down at 200*g* for 5 min at 4 °C.

v. Count the number of cells with a hemocytometer, and plate ~6,000 cells per well in a 96-well plate with 100 μl of culture medium.

vi. Incubate at 37 °C at low O_2 conditions for 24 h.

vii. After 1 d, prepare fresh osteogenic differentiation medium.

▲CRITICAL STEP Osteogenic medium must be prepared fresh at every medium change, but stocks of the reagents may be prepared in advance and frozen at −20 °C as shown in ‘Reagent setup’.

viii. Replace medium in all wells with osteogenic differentiation medium.

ix. Change medium every 2–3 d with fresh osteogenic medium.

x. After 14 d, aspirate off the medium and fix with 50 μl of 4% PFA at room temperature for 20 min.

xi. Prepare fresh 2% Alizarin red solution in water and filter through Whatman filter paper.

▲**CRITICAL STEP** The Whatman paper is folded into quarters and expanded into a funnel shape to be placed over a 50 ml conical tube to act as a filter.

xii. After fixation, wash the wells with water three times.

xiii. Add 50 μl of 2% Alizarin red to each well and incubate at room temperature for 30 min.

xiv. After incubation, remove the stain and wash the wells with water four times.

xv. After the fourth wash, incubate the wells in water for ~30 min to allow for excess stain to be removed.

xvi. Aspirate all water and allow the plate to dry for imaging and subsequent quantification of Alizarin red staining.

? TROUBLESHOOTING

(D) In vitro chondrogenesis ● Timing 2 weeks for expansion, followed by 2 weeks for differentiation—▲CRITICAL To ensure that after expansion there are sufficient numbers of hSSCs, hOPs or hCPs to have sufficient numbers for differentiation assays, at Step 22, sort at least 500 cells into a microcentrifuge

i. Transfer the entire contents of the microcentrifuge tube of sorted cells into a 10 cm tissue culture plate for expansion with 10 ml of culture medium. Swirl the plate gently to evenly distribute the cells.

ii. Incubate the cells at 37 °C at low O_2 conditions for 2 weeks with a medium change every 2–3 d.

iii. After expansion of at least 500 fresh sorted hSSCs, lift cells with 1 ml 0.05% trypsin and quench with two times the volume of complete MEMα medium.

iv. Centrifuge the cells at 200*g* for 5 min at 4 °C, remove supernatant and resuspend with 1 ml complete MEMα medium.

v. Count the number of cells using a hemocytometer and trypan blue, and centrifuge at 200*g* for 5 min at 4 °C.

vi. Resuspend at a concentration of ~3.5 million cells ml^{-1} in fresh human chondrogenic differentiation medium.

vii. Carefully place a 5 μl drop of the concentrated cells in the center of the well in a 48-well plate.

viii. Allow the plate to rest in the 37 °C incubator for 1 h.

ix. After 1 h, carefully add 300 µl chondrogenic differentiation medium to each well, taking care to disturb the micromass as little as possible.

▲**CRITICAL STEP** Chondrogenic medium must be prepared fresh at every medium change, but stocks of the reagents may be prepared in advance and frozen at −20 °C as shown in 'Reagent setup'.

x. Incubate for 14 d at 37 °C with low O_2 conditions, and medium change every 2–3 d with chondrogenic medium.

xi. After 14 d, aspirate off medium and fix with 100 µl 4% PFA for 20 min.

xii. Prepare fresh 2% Alcian blue solution in 0.1 M HCl.

xiii. After fixation, remove PFA and add 100 µl 2% Alcian blue to each well and stain for 30 min in the dark.

xiv. After staining, wash each well with 0.1 M HCl two times and then wash with water once.

xv. Allow the plate to dry for imaging and subsequent quantification of Alcian blue intensity if needed.

? TROUBLESHOOTING

(E) Single-cell analysis (SmartSeq2 protocol) ● Timing 1 week—▲CRITICAL

Single-cell complementary DNA libraries are constructed using the Smart-seq2 protocol with minor modifications[40]. Use cells from Step 22, sorted into 96-well plates and frozen. Plates can be stored frozen or used immediately.

i. Incubate plate at 72 °C for 3 min and place immediately back on ice.

ii. Add 6 µl of reverse transcription mixture into wells containing lysis solution. Briefly, cDNA from scRNA is generated by oligo-dT primed reverse transcription with MMLV SMARTScribe reverse transcriptase and a locked template-switching oligonucleotide.

iii. Prepare 15 µl $well^{-1}$ of PCR Preamp mixture and add to the existing 10 µl of the first strand reaction already in well. Run on the thermocycler as described in Table 2.

iv. Perfrom 25 cycles of PCR amplification, which is the optimal number of cycles determined to obtain cDNA concentration of at least 0.05–0.32 ng $µl^{-1}$ from each human skeletal lineage cell, using KAPA HiFi hotStart ReadyMix and ISPCR primers (Table 2)

v. Purify amplified cDNA using 0.7× volume Agencourt AMPure XP beads to remove oligos smaller than 500 bp following the manufacturers' instructions.

vi. Determine the resulting cDNA concentration and size distribution for each well is done on a capillary electrophoresis-based fragment analyzer, Advanced Analytical. On the basis of this analysis, normalize the cDNA concentration to

a desirable concentration range of 0.05–0.32 ng μl^{-1} by diluting the amplified cDNA from each well.

vii. Use the Nextera XT DNA Library Prep kit to tagment, uniquely barcode and PCR enrichthe cDNA following the manufacturers' instructions. Pool and purify libraries to remove fragments smaller than ~400 bp using 0.6× volume Agencourt AMPure XP beads.

viii. Quantified pooled libraries by a Bioanalyzer, using the High Sensitivity DNA analysis kit, and fluorometrically using Qubit dsDNA HS Assay kits and a Qubit 2.0 Fluorometer, and adjust the final concentration to 2 nM.

ix. Sequence the pooled libraries on NextSeq 500** to obtain 2× 151 bp paired-end reads at a depth of 1–4.5 × 10^6 reads $cell^{-1}$.

Troubleshooting

Troubleshooting advice can be found in Table 3.

Timing

Preparation of tissues and cells for flow cytometry

Tissue dissociation for fracture callus, Step 1A: 1–5 h

Tissue dissociation for adult femoral head or bone marrow aspirates Step 1B: 2–3 h

Tissue dissociation for fetal bone Step 1C: 1.5 h

IPSC differentiation Step 1D: 2–3 weeks

Red blood cell lysis Steps 2–3: 10 min

Isolation of cell populations from prepared samples by flow cytometry

Antibody staining, Steps 4–14: 1.5 h

Flow cytometry, Steps 15–22: 2 h

In vivo functional assessment

In vivo renal subcapsular transplant, Box 1: 1 h for procedure, 4 weeks for cell graft, 8 weeks for whole bone

Human xenograft microfracture model, Box 1: 1 h for procedure, 6 weeks for engraftment

In vitro functional assessment

In vitro colony formation, Step 23B: 2 weeks

In vitro osteogenesis, Step 23C: 2 weeks for expansion, followed by 2 weeks for differentiation

In vitro chondrogenesis, Step 23D: 2 weeks for expansion, followed by 2 weeks for differentiation

Step 23E, Single-cell analysis: 1 week

Box 2, Lentiviral transduction of hSSCs: 4 d

Anticipated results

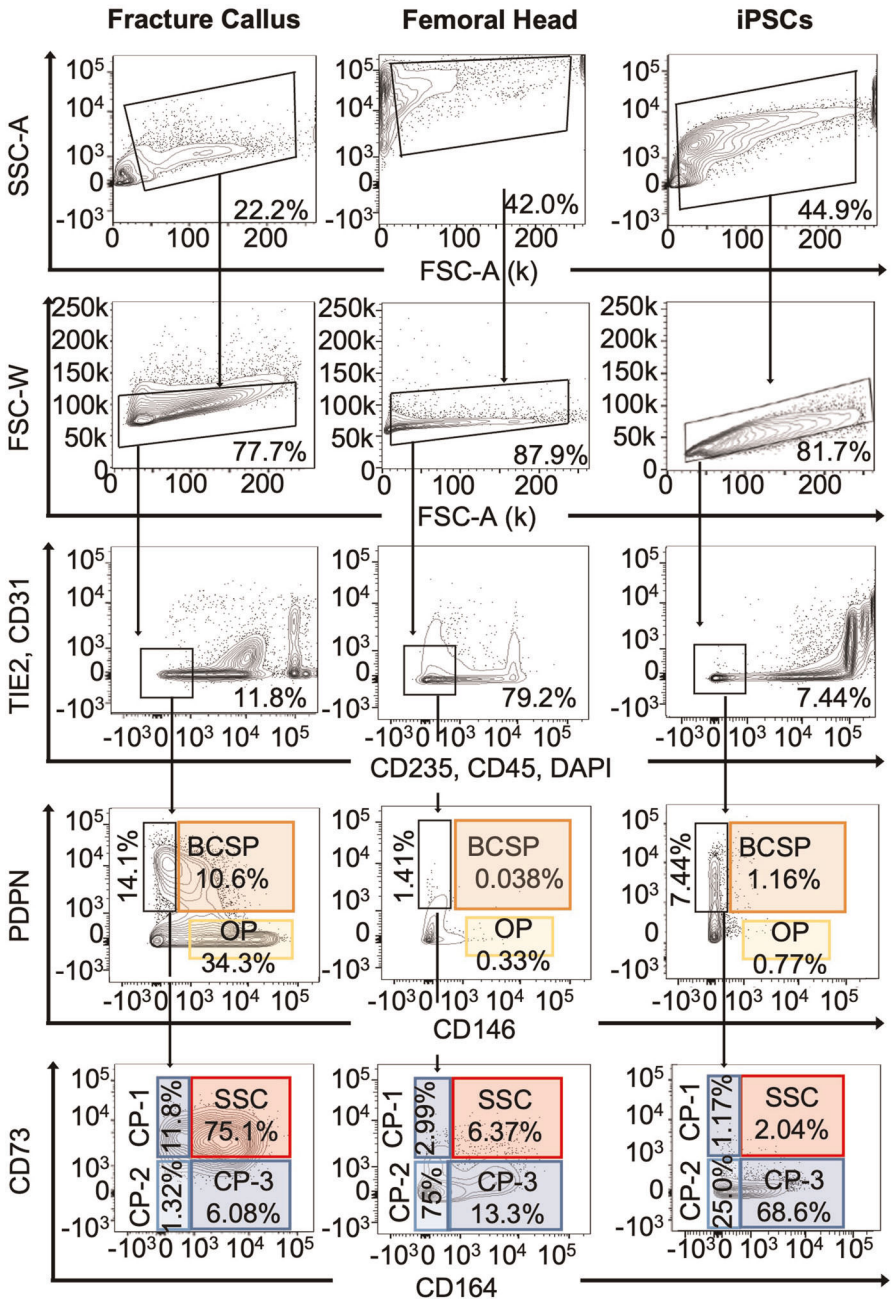
Properly executed, this protocol should lead to the isolation and functional assessment of hSSCs and downstream progenitors derived from different tissue and cell sources (Figs. 1–4). Starting with fresh tissue samples is essential to obtaining a reasonable amount of skeletal stem cells. Expect to obtain ~0.05–0.5% hSSCs, 0.5–5% hBSCPS, 0.25–2.5% hOPs and ~0.025–0.25% hCPs per 1 million events from samples; however, these percentages vary depending on the quality and type of samples. Cell yield is dependent on multiple factors, such as tissue source, anatomical sublocalization, age, sex, potential patient comorbidities, etc., and therefore is generally hard to predict. As a rule, a 0.125 cm^3 piece of starting tissue will allow for the isolation of >20,000 of hSSCs from fetal bones, >1,000 hSSCs from fracture specimens and >100 hSSCs from a femoral head (Extended Data Fig. 1). Also, as with mouse SSCs, the number of hSSCs significantly decreases with age, so expect fetal tissue to have the highest frequency of SSCs in comparison to specimens taken from older patients.

For our human xenograft model, the fetal tissue should be optimally engrafted after 6–18 weeks in mice (Fig. 3a). Of the three bone compartments, hSSC frequency will be highest in the cartilage (Fig. 3b). We expect the xenograft to intrinsically grow in the mouse at a developmental rate equivalent to that in humans in utero (Fig. 3c,d). The xenograft model can be utilized as a niche to study hSSCs in vivo, in which GFP-labeled SSCs can be injected into bone marrow compartment (Fig. 3e,f) and also analyzed upon microfracture injury. After microfracture, expect hSSCs to significantly amplify 1 week post-surgery.

Processing and digesting skeletal tissues will produce single-cell suspensions for flow cytometry, which is utilized to further separate the cells into the populations of interest for analysis. Flow cytometry-isolated cells can be transplanted in a renal kidney capsule for engraftment, plated for in vitro analysis or analyzed at the single-cell level. For in vivo renal capsule transplantations, a graft should begin forming as early as 1–2 weeks (Fig. 4c). It is important to note that the timepoint in which the graft is excised will dictate the type of tissue for analysis. For example, after 1 week post-transplantation, cartilage will be the primary tissue type and after 6 weeks, more bone tissue will have started forming due to the natural process of endochondral ossification. Also, expect fetal tissue to produce cartilage with more proteoglycans than adult cartilage. For the in vitro CFU-F assay, expect to obtain an average of 20–50 colonies per 200 cells (seeded at 10–50 cell cm^{-2}) depending on tissue source (Fig. 4e). hSSCs are also highly osteo- and chondrogenic, especially in fetal tissue specimens. Expect these cells to have strong staining of Alizarin red and Alcian blue after 2 weeks of differentiation.

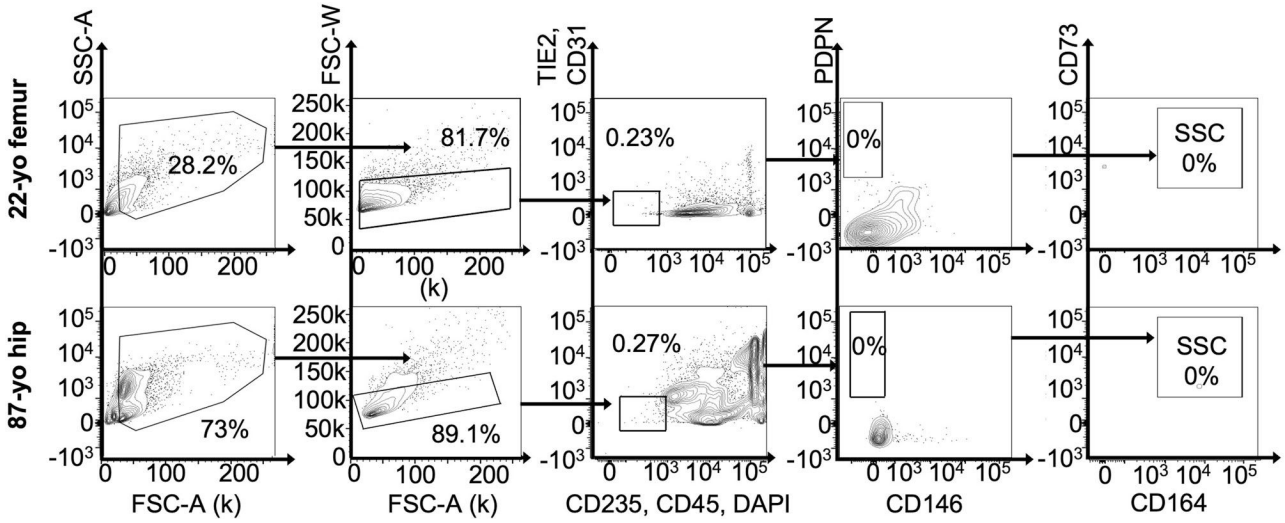
If conducted properly, more than 70% of all processed cells should generate high-quality transcriptomic data for analysis, i.e., comparable sequencing reads, at least 1,000 genes detected per cell, and low mitochondrial and ribosomal gene content. Proper RNAse-free working setup, FACS gating, sorting settings, and processing and clean-up steps are crucial prerequisite for success (Fig. 5b–d). We recommend using liquid-handling robots, if available, for consistent conditions throughout the protocol. We generally find that phenotypic hSSCs from different skeletal sites have high overall transcriptomic similarity and thus cluster quite closely when using Leiden clustering, independent of features used (Fig. 5e–h). Developmental state, age, sex and comorbidities of patients usually contribute to higher transcriptomic diversity among hSSC sources. It is worth noting that, compared with skeletal stem cells from inbred mice, a patient source might drive the distinct clustering of hSSCs[31,35]. In our experience, this can be mitigated by increasing the number of patient samples, together with appropriate batch correction during downstream analysis. As mentioned above, despite high homogeneity within hSSCs of the same source, there is a degree of subclustering into specific cellular states that needs further dissection, in part with help of this approach, in the future (Fig. 5e–h).

Extended Data

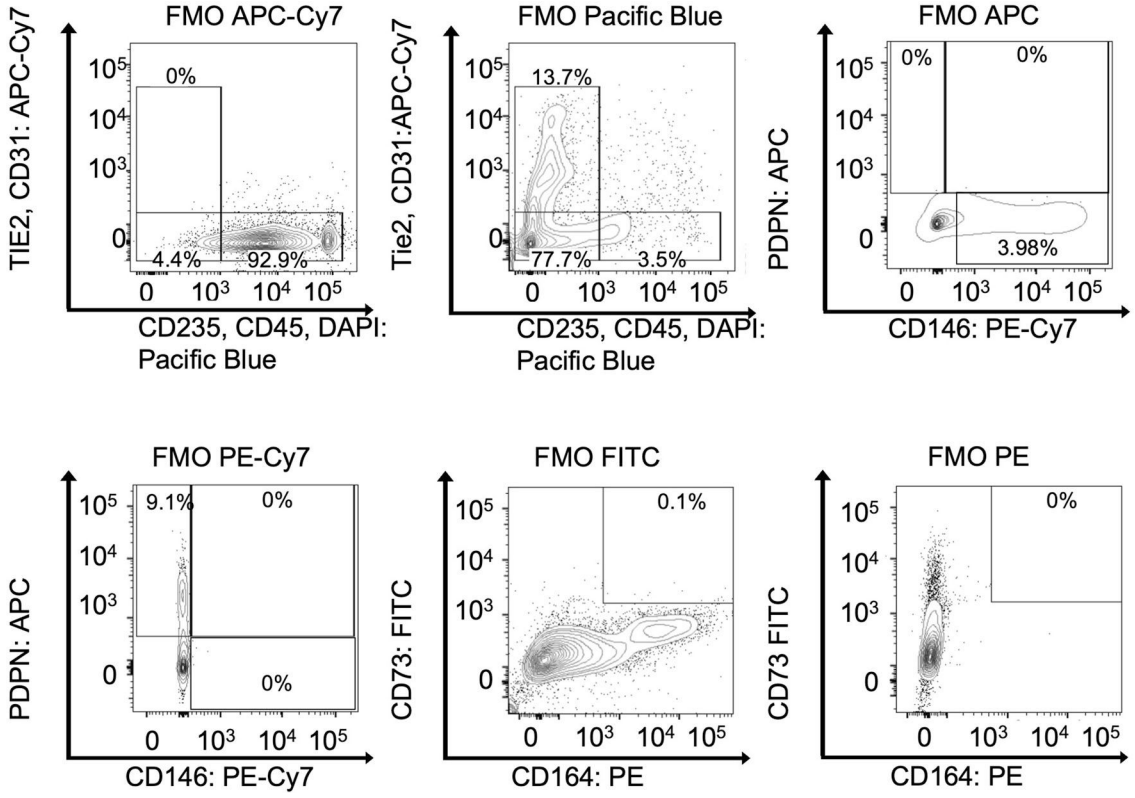


Extended Data Fig. 1 |. FACS gating strategy for other hSSC sources.

FACS gating plots for other three hSSC sources, including: fracture callus (28-year-old clavicle), femoral head (87-year-old) and iPSCs (human-monocyte-derived line, SCVI 113).PRO



Extended Data Fig. 2 |. FACS gating strategy for SSCs of bone marrow reamings.
FACS gating plots for SSCs collected from human bone marrow reamings of 22-year-old femur (top) and 87-year-old hip (bottom).



Extended Data Fig. 3 |. Fluorescence Minus One (FMO) gating strategy.
FMO controls help to define the negative signal for the given antibody and allow for a more informed gating strategy. Cells were isolated from a 76-year-old male femoral head. Representative plots were generated on a FACS Aria II for each FMO (eg. anti-Tie2/CD31

in APC-Cy7, anti-CD235/CD45/DAPI in Pacific Blue, anti-PDPN in APC, anti-CD146 in PE-Cy7, anti-CD73 in FitC, and anti-CD164 in PE).

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Data availability

All raw and processed scRNAseq data presented in this study have been submitted to the National Center for Biotechnology Information Gene Expression Omnibus online repository and are available under accession number GSE212609. We will share all other raw data upon request.

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Box 1 |

Human xenograft microfracture model

Procedure

Subcutaneous human bone xenograft ● Timing 6 weeks

!CAUTION All experiments involving mice should be performed in accordance with relevant guidelines and institutional regulations.

1. Anesthetize P3–P7 immunocompromised NSG pups by placing the pup in an induction chamber that is under or above a safe heat source. The flow of oxygen should be at 2 l min^{-1}, then begin the flow of isoflurane at a concentration of 3%. Once the breathing pattern has slowed, check for the absence of the righting reflex or toe pinch response.

   ▲**CRITICAL STEP** An NSG mouse should be used as the recipient to avoid immune rejection. Neonatal pups also have higher engraftment and vascularization of xenograft than older mice.

2. Transfer the mouse to a nose cone that has an oxygen flow rate of 1–2 l min^{-1} and an isoflurane rate of 2–3%. A safe heat source should be provided, such as a homeothermic blanket, and the respiratory rate should be closely monitored. Adjust the isoflurane concentration as necessary.

3. Administer analgesia at the appropriate dosage for the weight of the pup. Note: indomethacin (2 mg kg^{-1}) injected via intraperitoneal injection once per day for 7 consecutive days is recommended.

4. Treat the dorsal area with 70% ethanol and povidone–iodine solution.

5. With forceps, grasp the skin just below the head of the pup and use scissors to make a 2–3 mm incision. Gently spread the skin from the muscle using blunt dissection forceps.

6. Carefully insert the phalange (isolated as described in Step 1C iv) under the skin down along the dorsum.

7. Close the incision with 6–0 simple interrupted sutures and treat the area with 70% ethanol.

8. Transfer the pup to a prewarmed recovery cage and follow institutional instructions for postoperative care.

9. Before progressing to microfracture surgery, wait at least 6 weeks to ensure optimal engraftment.

Human xenograft microfracture surgery ● Timing 30 min

10. Anesthetize and prepare the xenograft mouse for surgery using the methods described in steps 1–3 above.

11. Using forceps, grasp the skin on the dorsal area and carefully make an incision next to the phalange.

12. Gently hold the phalange in place with blunt forceps while using a drill to make the microfracture defects along diaphyseal cortical surfaces of the phalangeal bone. Additionally, hSSCs can be transplanted into defect sites (Step 23A(i–iii) describes cell preparation).

13. Close the incision with 6–0 simple interrupted sutures and treat the area with 70% ethanol.

14. Transfer the mouse to a prewarmed recovery cage and follow institutional instructions for postoperative care.

Box 2 |

Lentivirus infection of SSC ● Timing 4 d

1. After expansion of hSSCs as directed in Step 23B, lift cells with 0.05% trypsin and quench with two times the volume of culture medium.
2. Spin down at 200*g* for 5 min at 4 °C. Count the number of cells, and plate ~70,000 cells per well in a 12-well plate with 1 ml of culture medium. Alternatively, fresh sorted cell can also be directly plated in 12-well plate at density of 5,000 cell per well without expansion.

   ▲**CRITICAL STEP** A six-well plate cannot be used for this spin infection protocol as half of the well will not be covered by the medium during the spin. Incubate at 37 °C for 2 d until the cell confluent reaches 30–50% before proceeding to the next step.
3. Incubate at 37 °C for 24 h, until the cells reach 30–50% confluency. Alternatively, if fresh sorted cells are being used, incubate at 37 °C for 2 d until cells reach 30–50% confluency.
4. Thaw 20 μl 100× concentrated lentiviral supernatant aliquot on ice, add it to room temperature Opti-MEMI Reduced Serum Media with final expected mixture volume at 1ml $well^{-1}$, add 1μl Lipofectamine(polybrene) per ml of expected final volume and gently mix with a pipette.

   ▲**CRITICAL STEP** Virus titration is recommended before actual experiment to avoid cell death caused by overdose of virus.
5. Remove medium from 12-well plate(s) and add 1 ml per well of the mixture from step 4.
6. Wrap the plate(s) in Saran Wrap with an equivalent balance plate. Spin the plate in plate carriers at 1,500*g* for 60 min at 24–27 °C.
7. Dilute the lipofectamine/polybrene with 3 ml of fresh culture medium in each well and incubate the cells at 37 °C.
8. Change medium 24 h after infection. Fluorophore signal should be detectable from infected cell 3 d after spin infection.
9. Purify the infected cell based on the fluorophore intensity by FACS sort for further experiments listed in downstream assay.

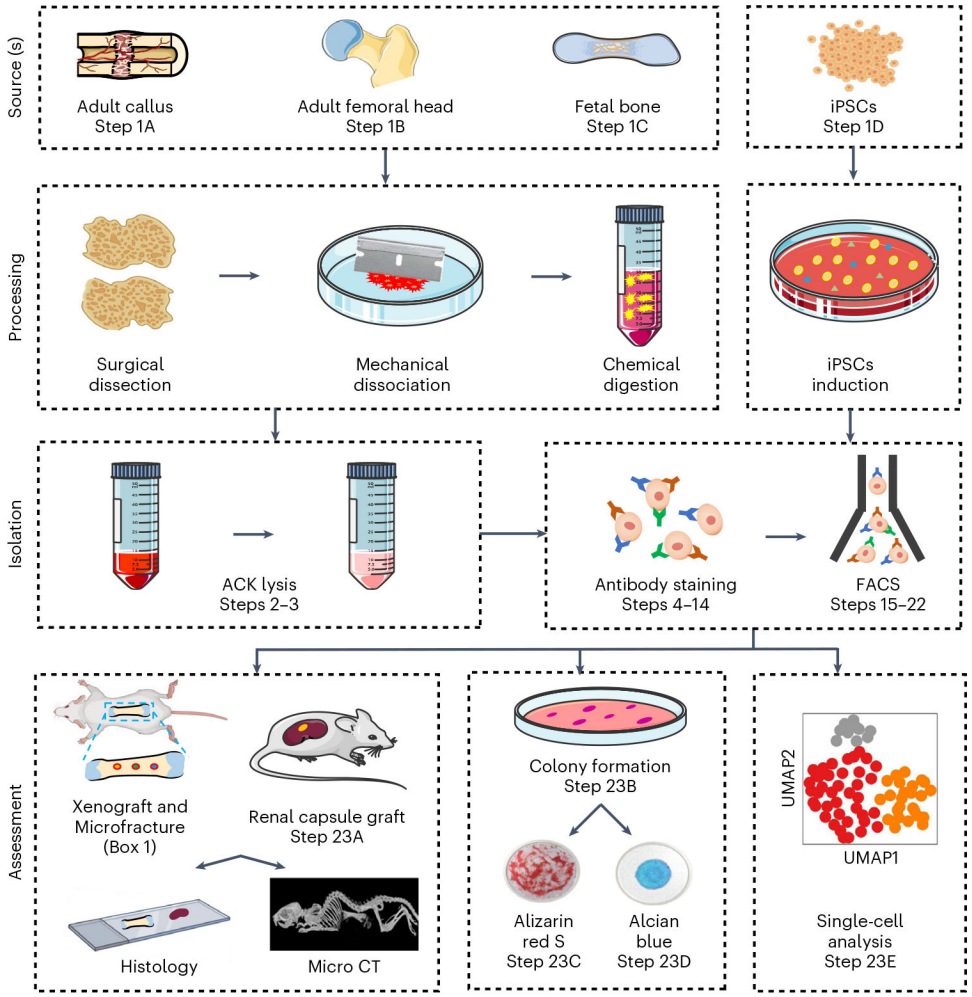

Fig. 1 |. Overview of the protocol for human skeletal stem cell isolation and functional assessment.

hSSCs can be isolated from multiple skeletal tissues and induced from pluripotent stem cells. The protocol includes isolation of the human skeletal elements by tissue dissection (Step 1A–D); dissociation of the skeleton into a single-cell suspension by mechanical (Step 1A–D) and enzymatic digestion (Steps 1A–D); depletion of red blood cells with ACK lysis buffer (Steps 2–3); antibody staining of the cells for skeletal lineage surface markers (Steps 4–14) and analysis and sorting of the cells on a flow cytometer (Steps 15–22); functional assessment of skeletal-lineage phenotype by in vivo xenograft (Box 1), renal subcapsular transplantation (Step 23A), in vitro colony-formation assay (Step 23B) and scRNAseq (Step 23E; UMAP, uniform manifold approximation and projection); analysis of grafts by micro CT, Movat Pentachrome and immunohistochemistry, and analysis of differentiation cultures by crystal violet (Step 23B), Alcian blue staining (Step 23C) or Alizarin red S staining (Step 23D).

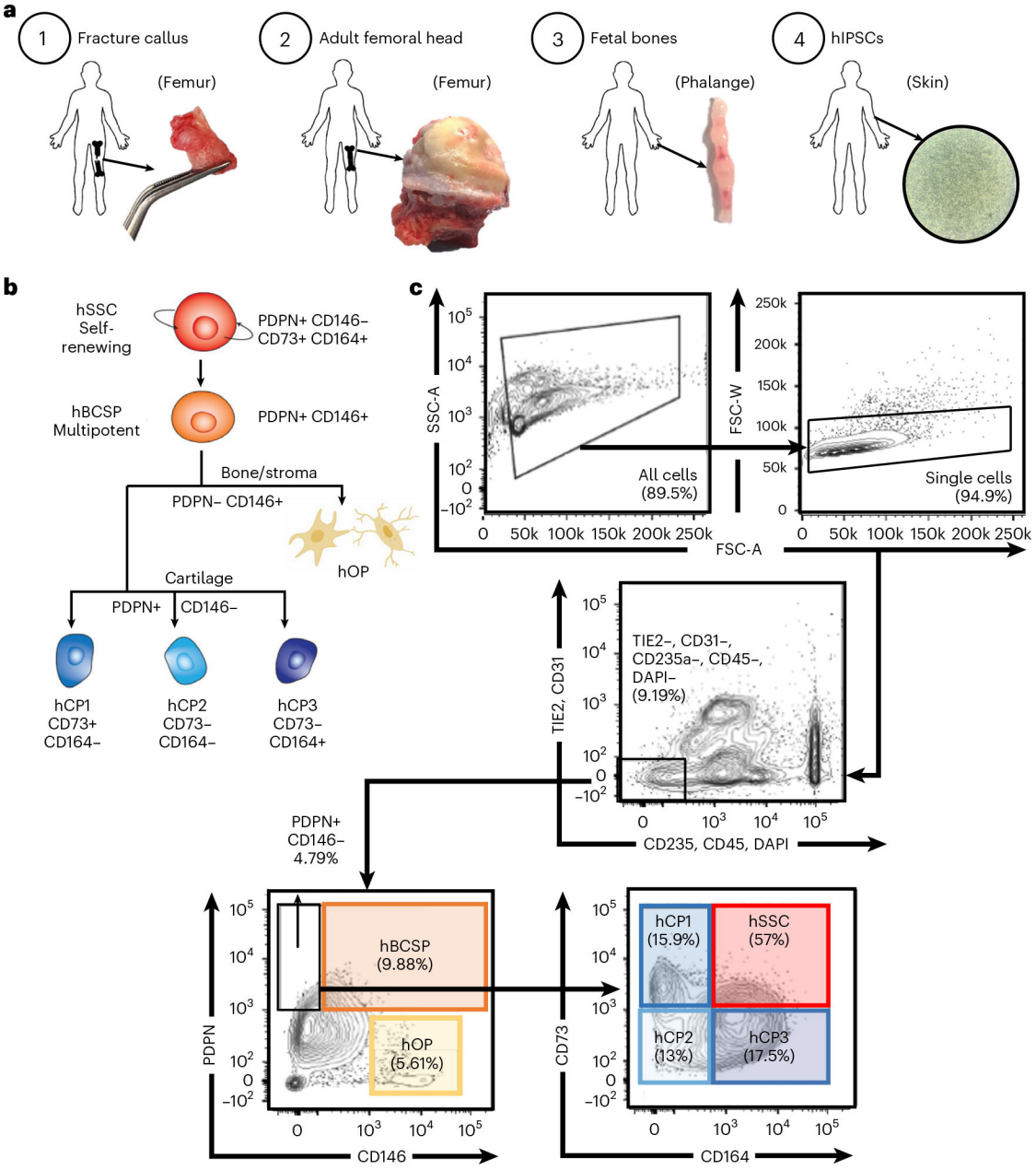

Fig. 2 |. hSSC sources, hierarchy and FACS gating strategy.

a, Representative sources of hSSCs: Fracture callus tissue of a 40-year-old male patient, femoral head of a 76-year-old patient, phalange bones of a 17-week-old fetus (gestational age) and iPSC-derived SSCs 2 weeks after induction (brightfield image). **b**, hSSC hierarchy and associated markers. The self-renewing hSSC gives rise to the multipotent hBCSP cells. The hBCSP may differentiate into either one of three chondroprogenitor populations (hCP1–3) responsible for cartilage formation, or the hOP responsible for bone and stroma formation. **c**, Representative FACS plots for human fetal bone sample. Variation between SSC and progenitor yields vary between sample tissue source and due to external factors, such as patient age, time since injury and patient health.

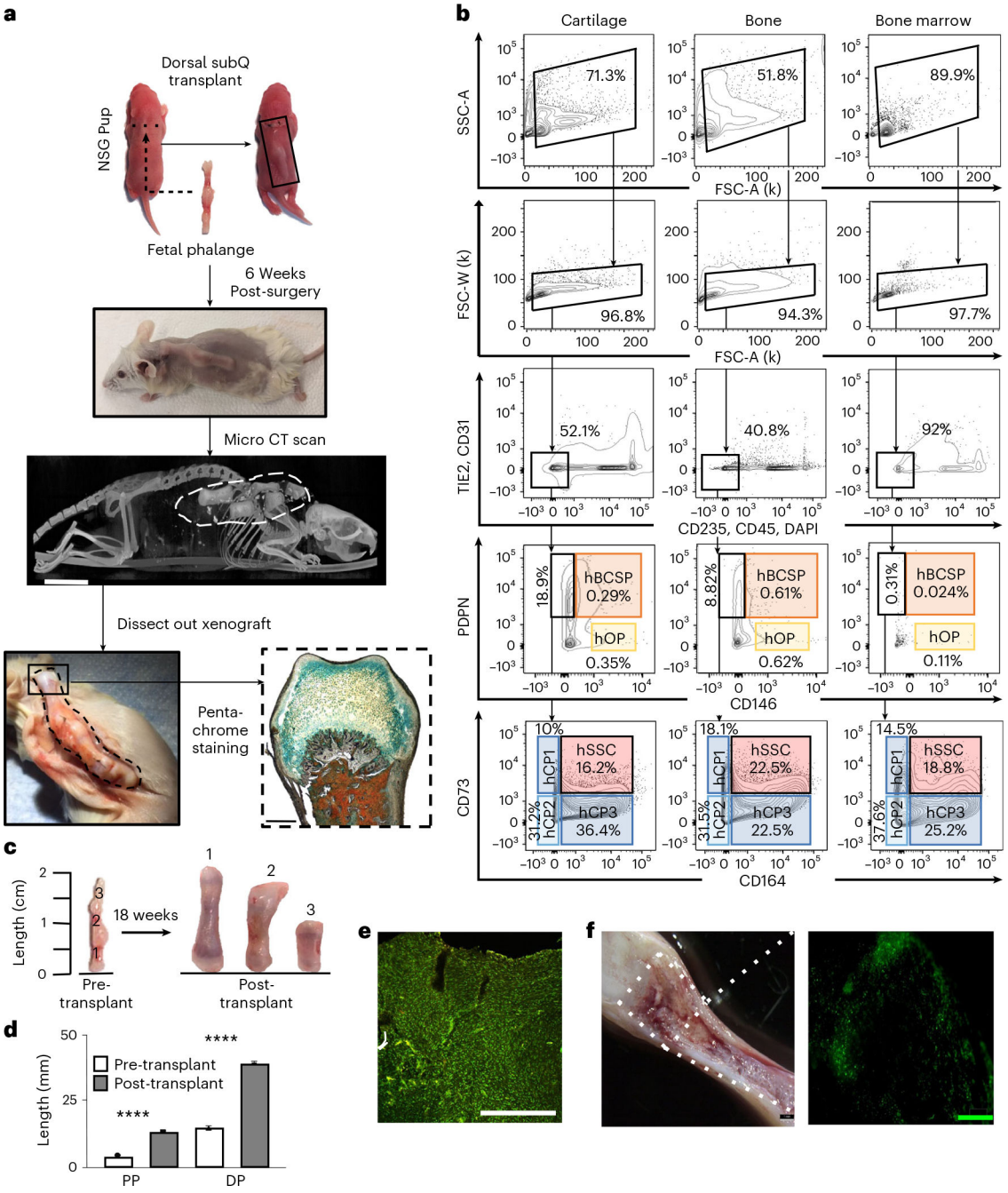

Fig. 3 |. Subcutaneous human xenograft model and FACS isolation of hSSC lineages.

a, Phalange from a 17-week-old fetus is subcutaneously transplanted onto dorsum of a postnatal day-3 pup. Six weeks post-transplant, the fetal xenograft is subjected to micro CT, and then excised out of mouse for pentachrome staining. **b**, Representative FACS plots from three tissue components of the human fetal phalange xenograft: cartilage, bone and bone marrow. **c**, Gross images of human fetal phalanges pre- and post-implantation after 18 weeks. 1 = proximal phalanx, 2 = middle phalanx, 3 = distal phalanx. **d**, Quantification of length of proximal phalanx (PP) and digit (DP) shown in (**c**). (*n* = 3 specimens per

group). Graphs show mean ± s.e.m. Two-tailed Student's *t*-test. ****≤000005. **e**, Human nuclear staining (HNA) of fetal phalange subcutaneously transplanted. **f**, Xenografted fetal bone intramedullary injected with lentivirally GFP-labeled patient-derived hSSCs dissected in half. Inset: magnified image of GFP signal from lentivirally labeled hSSCs. Scale bars: 200 mm (**a**), 200 mm (**e**) and 1 mm (**f**).

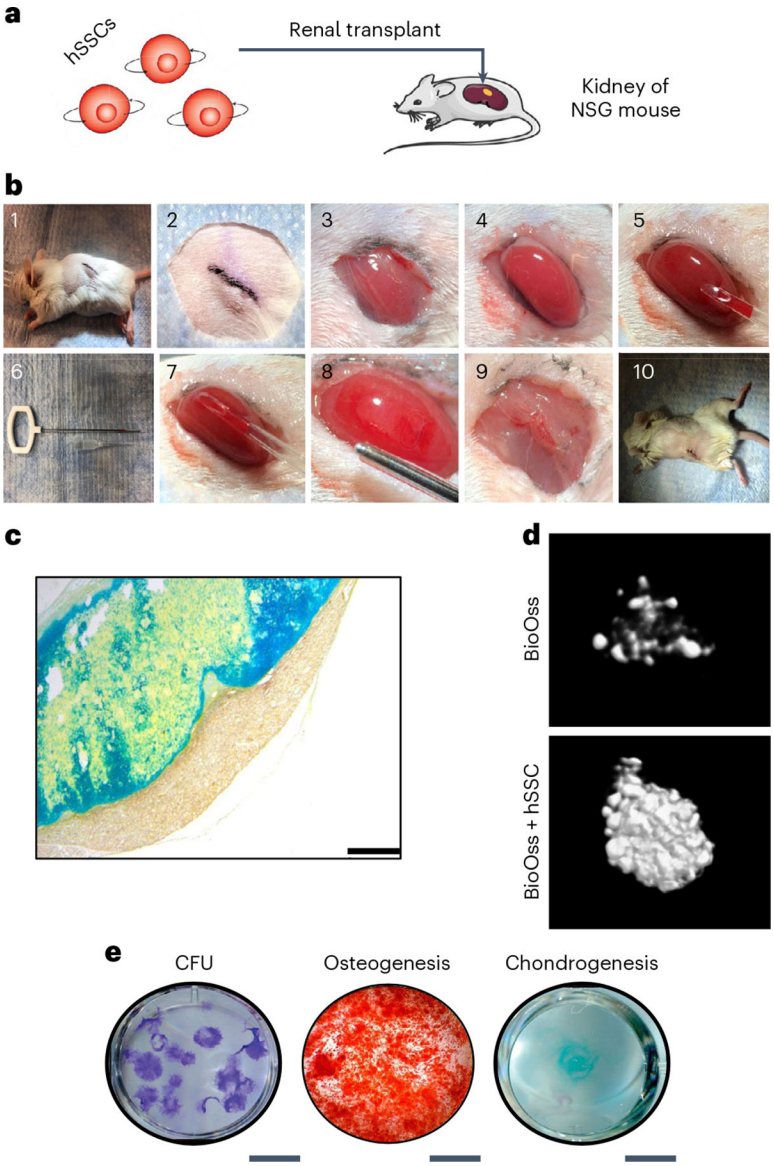

Fig. 4 |. Renal capsule xenograft model and in vitro skeletal differentiation assays.
a, Schematic of hSSC renal and intra-xenograft transplants. **b**, Step-by-step procedure of renal capsule transplant of hSSCs in NSG mice (details in Step 23A). **c**, Pentachrome staining of hSSC-derived grafts 4 weeks after transplantation beneath the renal capsule. **d**, Representative micro CT images of a patient-derived hSSC graft that was co-transplanted with Matrigel and BioOss (anorganic bovine cancellous bone; bottom) and a BioOss transplant without cells as control (top). **e**, In vitro assays: CFU-F test for clonogenicity of hSSCs stained by crystal violet, osteogenesis differentiation assay for bone formation potential stained by Alizarin red S and chondrogenesis differentiation assay for cartilage forming potential stained by Alcian blue. Scale bars: 1 mm (**c**) and 10 mm for CFU, 200 μm for osteogenesis and 5 mm for chondrogenesis (**e**).

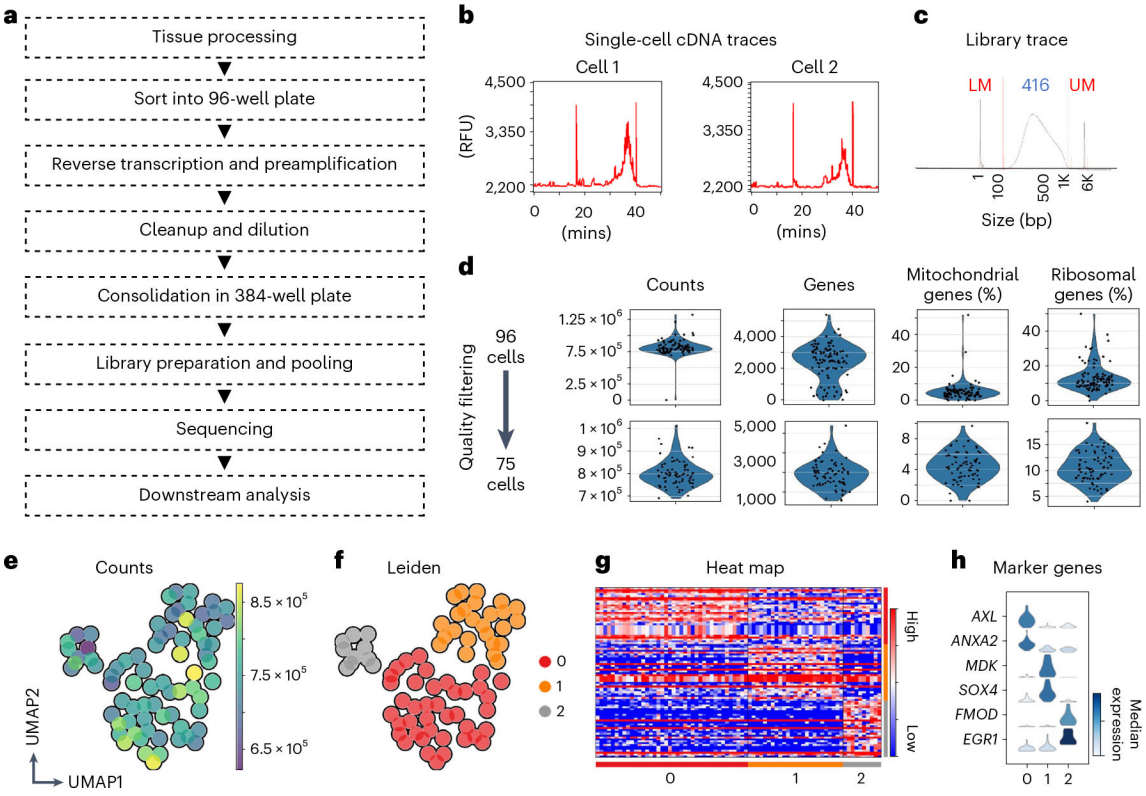

Fig. 5 |. ScRNAseq platform for analyzing hSSCs.

a, Step-by-step platform for processing hSSCs using SmartSeq2 and subsequent downstream analysis. **b**, Representative graphs of single-cell cDNA traces. **c**, Representative graph of pooled library trace using a Bioanalyzer. **d**, Example of quality filtering of 96 sequenced single cells of vertebral fracture hSSCs from a 40-year-old man for total RNA counts, number of genes expressed, percentage of mitochondrial genes and ribosomal genes. **e**, Representative UMAP clustering graph at resolution of 0.4, n_neighbors = 10 and n_pcs = 8 (based on principal component analysis elbow plot). **f**, Corresponding Leiden clustering graph. **g**, Representative heat map of top 30 genes for each cluster. **h**, Cluster marker genes that can be used for further separation and analysis of hSSC heterogeneity.

Table 1 |

Antibody staining concentrations

Antibody	Ratio
Tie2 – Biotin	1:50
CD31 – Biotin	1:50
CD45 – Pacific blue	1:50
CD235 – Pacific blue	1:50
CD146 – PE-cy7	1:50
PDPN – APC	1:50
CD73 – FitC	1:50
CD164 – PE	1:50
Secondary: Streptavidin APC-AF750	1:100

Table 2 |

ScRNAseq thermocycler steps

		Step	Temperature	Time	Cycle
Reverse transcriptase					
Lid	105 °C	Step 1	42 °C	90 min	
Vol	10 μl	Step 2	70 °C	5 min	
Run time	1 h, 37 min	Step 3	4 °C	∞	
Preamplification of cDNA					
Lid	105 °C	Step 1	37 °C	30 min	
Vol	25 μl	Step 2	98 °C	3 min	
Run time	2 h, 37 min	Step 3	98 °C	20 s	
			67 °C	15 s	23
			72 °C	6 min	
		Step 4	72 °C	5 min	
		Step 5	4 °C	∞	

Table 3 |

Troubleshooting table

Step	Problem	Possible reason	Solution
1A(x), 1B(viii), 1C(x)	Sample does not appear cloudy after shaking	Insufficient digestion	Process the sample into smaller pieces, check pH of collagenase, increase time of digestion
3	Sample still red after centrifugation	Not enough ACK lysis buffer used for the sample	Repeat another round of ACK lysis if the sample is excessively bloody or large
23A(xxiii)	Cell graft diffusion Graft found outside the capsule Graft is missing	Matrigel is not solid Graft transplanted under the fat layer instead of capsule Graft has dropped off the kidney	Move the transplant to room temperature for at least 5 min Make a deeper cut on the kidney to make sure insert the 20 μl tip under the right layer Make a smaller cut on the capsule, and add sponge after transplants to avoid grafts from dropping off from the kidneys
	Graft did not form bone	hSSC starting number too low Age of donor No BioOss added	Use a higher number of hSSCs Use a younger patient donor Add BioOss
23C(xvi), 23D(xv)	No staining detected	Reagents no longer effective	Avoid multiple freeze/thaws of reagents, prepare in small volume aliquots
		Too few cells plated initially Cell layer has lifted because too many cells plated initially	Plate for differentiation at a higher density Plate for differentiation at a lower density