Materials and Methods

Cell culture

H1 and H9 hPSCs, NC3-1, IMR904, UC01 (*SOX9-IRES-tdTomato*), and *AkaLuc-BsdR* UC01 (*SOX9-IRES-tdTomato*) hPSCs were routinely propagated in Pluripotency Growth Master 1 (PGM1, Cellapy, Cat# CA1007500) medium on cell culture plates coated with Matrigel (Corning, Cat# 354230 or MeisenCTCC Cat#MS0101ZY). The working solution of Matrigel (MatriWS) was prepared using DMEM/F12 (Gibco, Cat# C11330500) at 1mg/12 ml, and the coating was conducted at 37 ℃ for at least one hour. All the hPSC lines were maintained carefully to avoid any spontaneous differentiation. Cell splitting was carried out at 1:5 with 0.5 mM EDTA (ThermoFisher, Cat# AM9260G) in PBS (Gibco, Cat# C10010500BT) when the confluency reached 80%. The cells should be lifted from plates as fine clumps, and the ROCK inhibitor was excluded during routine culture unless otherwise mentioned. Human bone marrow stem cells (hBMSCs) were cultured in MEM alpha (Gibco, Cat# 12571063) supplemented with 20% v/v lot-selected non-heat inactivated fetal bovine serum (FBS) (Hyclone, Cat# SH30084.03) and 1% v/v penicillin/streptomycin (pen-strep, Gibco, Cat#4e T0665). The cells were passaged with 0.25% trypsin-EDTA (Thermofisher, Cat# 25200056) when the confluency reached ~70% and replated at the ratio of 1:5.

UC01 (*SOX9-IRES-tdTomato*) hPSC line construction

Knock-in was achieved by non-homologous mediated end joining using the CRISPR/Cas9 system with guide RNAs (gRNAs) targeting the 3' untranslated region (UTR) of *SOX9* (Figure S1A). Four gRNAs were designed using the online tool CRISPR[1], with PAM sequences located within 100 base pairs (bp) downstream from the stop codon of *SOX9*. The targeting vector containing the *IRES-tdTomato-PGK-PuroR* cassette was synthesized by BGI Group, Beijing. The gRNAs were annealed and subcloned into both the PX330 vector harboring the Cas9-expressing cassette and the targeting vector. To generate the UC01 (*SOX9-IRES-tdTomato*) hPSC line, parental hPSCs were seeded sparsely (~40% confluency) as single cells on MatriWS-coated 12-well plates. The next day, the cells were co-transfected with 800ng PX330-*SOX9* gRNA and 500ng *IRES-tdTomato-PGK-PuroR* donor vector by Lipofectamine Stem Transfection Reagent (ThermoFisher, Cat# STEM00015) using manufacturer's protocol. Drug selection was carried out using puromycin for 10 days till the confluency reached 80%. The concentration of puromycin increased gradually from 0.2 to 0.5μg/ml and was kept constant at 0.5μg/ml for subsequent colony screening. To pick correct colonies, the transfection pools were dissociated into single cells by Accutase (Innovative Cell Technologies, Cat#AT-104) and seeded at a density of 6-10 cells/well in MatriWS-coated 96-well plates and cultured with PGM1

supplemented with 0.5μg/ml puromycin and 10μM ROCK inhibitor, Y27632 (MCE, Cat#HY-10071). This density would result in ~ 15% single colonies in each plate, which were propagated further till sufficient cells could be harvested for genotyping. To verify correct integration, both 5' and 3' ends were amplified by PCR and subject to Sanger sequencing. gRNAs used in this study were listed below:

gRNA names	Sequence with *PAM* (5' to 3')
gRNA #1	CCTCCCACGAAGGGCGAAGA*TGG*
gRNA #2	CCATCTTCGCCCTTCGTGGG*AGG*
gRNA #3	TGTCCAAAGGGAATTCTGGT*TGG*
gRNA #4	AAAAATAACCGAAGAAAGAG*AGG*

Generation of *SOX9*$^{+}$ sclerotomal progenitors (scl-progenitors)

Before differentiation, routinely cultured hPSCs were re-split to another MatriWS-coated plate. To control the starting cell density, the cells were detached from culture plates by 0.5mM EDTA as fine clumps when the confluency reached 80% and split at 1:5. Once plating, vigorous shaking should be done several times to make sure the clumps were evenly distributed across the culture plates. The next day, fine and separated colonies should be present on the culture plates and any signs of over-confluency represented by large-sized integrated multi-colonies should be avoided to maintain differentiation efficiency.

All differentiation was conducted in two-dimensional serum-free conditions using a modified chemically defined medium with insulin (CDMi)[2]. To prepare CDMi, IMDM (Gibco, Cat# 31980-097) and F12 (Gibco, Cat# 31765-092) were mixed at 1:1 supplemented with 1% v/v concentrated lipids (Gibco, Cat# 11905-031), 450μM monothioglycerol (Sigma, Cat# M6145), 0.7μg/mL insulin (Solarbio, Cat# I8830), 15μg/ml transferrin (Sigma, Cat# T0665), 1% v/v pen-strep, and 1 mg/mL polyvinyl alcohol (Sigma, Cat# P8136-250G). The polyvinyl alcohol was first solved at 25mg/ml in ultrapure water (UP H_2O) by heating to 85℃ and then cooled on ice before being added to the medium.

To start differentiation, day-0 hPSCs were gently washed with the wash medium (formulated as 3mg/ml bovine serum albumin (BSA) in DMEM/F12) and were induced to primitive streak (PS) in CDMi containing Activin A (30ng/ml, Solarbio, Cat#P00101), CHIR99021 (7μM, MCE, Cat#HY-10182) and bFGF (20ng/ml, Peprotech, Cat#100-18B) for 24 hours; the next day, day-1 PS was washed with wash medium and differentiated to presomitic mesoderm (PSM) in CDMi containing SB431542 (20μM, MCE, Cat#HY-10431), CHIR99021 (3μM), LDN193189 (125nM, MCE, Cat#HY-12071A), and bFGF (40ng/ml) for another 24 hours; subsequently, the day-2 PSM was dissociated to single

cells by TrypLE (Gibco, Cat#12605028) and four-fold volume of wash medium was used to stop digestion. The cells were then pelleted by centrifugation at 300g for 3 min and resuspended for further wash and cell counting. Next, cell suspension was re-collected by centrifugation again at 300g for 3 min and resuspended in CDMi supplemented with SAG (200nM, MCE, Cat#HY-12848), LDN193189 (600nM), XAV939 (0.5μM, MCE, Cat#HY-15147), and Y27632 (10μM) for the following *SOX9*$^{+}$ scl-progenitor differentiation. The cells were seeded onto another MatriWS-coated plate at the density of $1x10^5$ cells/cm^2. Also, vigorous shaking should be done several times immediately after plating to make sure the cells are evenly distributed across the culture plates. The cells were cultured for 24 hours where the medium was replaced with CDMi without Y27632 for another 24 hours of induction. (Note: the plating density of PSM could be variable for different hPSC lines. Cell death may occur when cells are plated too sparsely, while overcrowding may cause unintended and nonuniform differentiation. Optimization should be done when changing the lines).

For the traditional differentiation (Figure S2A), the induction of PS and PSM was kept the same as mentioned above. After being washed with wash medium, the day-2 PSM was differentiated to somite (SM) in CDMi containing XAV939 (0.5μM) and PD0325901 (1μM, MCE, Cat#HY-

10254) for 24 hours; the following day, the SM was washed with wash medium before induced to sclerotome by CDMi containing SAG (200 nM) and LDN193189 (600nM) for 72 hours with medium changed every day. The final *SOX9*$^{+}$ scl-progenitors were obtained by fluorescence-activated cell sorting (FACS).

Quantitative PCR

Total RNAs were extracted from cells using TRI Reagent (Sigma-Aldrich, Cat# T9424) and then reverse-transcribed to cDNA with the PrimeScript RT reagent kit with gDNA Eraser (TAKARA, Cat# RR047A) according to the manufacturer's protocol. The quantitative PCR was performed using a CFX384 Optics Module (Bio-Rad, USA) with AceQ Universal SYBR qPCR Master Mix (Vazyme, Cat# Q511-02), and the relative expression levels were calculated with the comparative threshold cycle (ΔΔCT) method. All tests were performed in triplicate. Primers are listed in Table S1.

Immunocytochemical staining

Adherent cells cultured on a cover glass or confocal dish were fixed with 4% paraformaldehyde (PFA) for 8 min at room temperature (RT) before being washed twice with PBS. Permeabilization was conducted for intramembranous antigen with 0.3% PBST (v/v Triton X-100 in PBS),

followed by blocking with blocking buffer (5% bovine serum albumin (BSA, Sigma, Cat# A3311-10G) in PBS) at RT for 1hr. Subsequently, the cells were incubated with primary antibodies diluted in blocking buffer overnight at 4℃ and then were washed three times with PBS. Next, the cells were stained with secondary antibodies in the dark at RT for 1hr and were washed again with PBS. For cells cultured on cover glass, the samples were mounted using the mounting medium with 4,6-diamidino-2-phenylindole (DAPI) (ZSJB-BIO, Cat# ZLI-9557) for nuclear staining. For cells cultured on the confocal dish, the nuclei were stained by Hoechst 33342 (ThermoFisher, Cat#H3570). Immunofluorescent microscopy was conducted with Leica TCS SP5II and Olympus VS200.

Flow cytometry

For analysis or sorting *SOX9*$^{+}$ cells using the UC01 (*SOX9-IRES-tdTomato*) cell line, the cells were first dissociated into single cells by Accutase and were washed once with 5% BSA. The cells were then resuspended in PBS and filtered with a 70-μm strainer for downstream experiments. Day-0 hPSCs were used as the negative control.

For intracellular or cell-surface protein staining, cells were dissociated into single cells by Accutase and were counted with Cell Counter (ThermoFisher). 1x10^{6} cells were used for a single test. Permeabilization

was performed using 0.3% PBST for intracellular antigens. The cells were then incubated with 100µl of primary antibodies (1:200 for SOX9 antibody and 1:100 for ITGA9 antibody) in 5% BSA at RT for 40 minutes and were then washed twice with 5% BSA. The cells were subsequently stained with 100µl of secondary antibodies (1:400) in 5% BSA at RT for 30 min protected from light and washed twice with 5% BSA. Samples stained with isotype IgG were used as the negative control. All the analysis and sorting were done by BD Fortessa and Aria III (BD Biosciences, USA).

Generation of Chondroprogenitors from *SOX9*$^{+}$ scl-progenitors

Adherent cells were first dissociated into single cells by TrypLE, and sorted cells were collected by centrifugation at 300g for 3 min. After being washed once with wash medium, $1.5x10^5$ cells were collected in the U-shaped ultra-low attachment 96-well plate, suspended in CDMi supplemented with 200nM SAG, 600nM LDN193189, and 10µM Y27632 followed by centrifugation at 300g for 6min to form spheroids overnight. After being washed once with wash medium, the spheroids were cultured by chondrocyte induction (CI) medium formulated as DMEM supplemented with 0.1µM dexamethasone (DEX) (MCE, Cat#HY-14648), 50µM ascorbic acid (Sigma, Cat#A8960), 50µg/ml L-proline (Sigma-Aldrich, Cat#P0380), 20ng/ml TGFβ3 (Peprotech, Cat#100-36E), 20ng/ml BMP2 (Peprotech, Cat#120-02), 20ng/ml bFGF, 1% v/v ITS+1 (Sigma-

Aldrich, Cat#12521-5ML), 1% v/v sodium pyruvate (Gibco, Cat#11360070), and 1% v/v pen-strep for indicated days.

Osteogenic induction *in vitro* for Alizarin red staining

Adherent cells were dissociated into single cells by TrypLE, and sorted cells were collected by centrifugation at 300g for 3min. After being washed once with wash medium, $1x10^5$ cells were seeded on a 96-well plate and cultured with α-MEM supplemented with 10% v/v FBS, β-glycerophosphate (10mM, Millipore, Cat#35675), DEX (0.1μM), ascorbic acid (50μg/ml) and 1% v/v pen-strep for 30 days.

Histological analysis for *in vitro* osteochondrogenic assays

Day-42 chondroprogenitors were fixed with 4% PFA overnight at 4°C. After being washed twice in PBS, samples were embedded in paraffin. Sections were made at 6μm using paraffin microtome (Leica RM2245) and were stained by Alcian blue, Safranin O, Toluidine blue O, Col II, Col I, Col X after rehydration in xylene and ethanol. For Alcian blue staining, 5% w/v Alcian blue 8GX (Sigma, Cat#A5268-100G) stock solution was first prepared in PBS and brought to 1% w/v working solution using 0.1N HCl. The sections were treated with 0.1N HCl for 2 min, followed by staining with Alcian blue working solution at RT for 30 min. After being washed in 0.1N HCl for 2 min, the sections were rinsed in ultrapure water to

remove any acidic content for subsequent nuclei staining by hematoxylin. For Safranin O staining, nuclei staining was first conducted with Wiegert's hematoxylin (Solarbio, Cat#G1371). The sections were then stained with 0.1% w/v Fast Green FCF (Sigma-Aldrich, Cat#F7252-100G, in UP H_2O) for 90s, followed by rinsing in 1% glacial acetic acid for 90s. Subsequently, the sections were stained with 5% Safranin O (Sigma, Cat#S2255-100G, in UP H_2O) for 1min. After rapid dehydration in 95% ethanol and 100% ethanol for 1min each, the sections were cleared in xylene and mounted with neutral resin. The Toluidine blue O staining was conducted using the manufacturer's protocol (Solarbio, Cat# G2543). For Col II staining, antigen retrieval was first carried out using pepsin (Sigma-Aldrich, Cat#R2283) for 30min at 37℃. The sections were then treated with 5% w/v BSA (Sigma-Aldrich, Cat#A9418-100G) and incubated with Col II antibody overnight at 4℃. After being washed in PBS three times, the subsequent secondary antibody staining and DAB reaction were conducted using the Universal IHC Staining Kit (ZSJB-BIO, Cat# PV6000) according to the manufacturer's protocols. The nuclei were stained with hematoxylin. The same procedures were used for COL I and COL X staining except that the heat-induced antigen retrieval was conducted using citrate buffer (pH 6.0).

Day 30 osteogenic cells were fixed with 4% PFA at RT for 8min and washed twice with PBS. The fixed cells were then stained by 2% w/v Alizarin red (Sigma-Aldrich, A5533) at RT for 2min, followed by washing three times with PBS.

Comparative evaluation assays for chondroprogenitor generation

Current protocol vs. reference methods

For the current protocol, the chondrospheroids were generated using *SOX9*$^{+}$ scl-progenitors by centrifugation as mentioned above, and were cultured in the CI medium for 4 days. For reference method I[3], the *SOX9*$^{+}$ scl-progenitors were induced to chondroprogenitors in CDMi containing 20 ng/ml BMP4 for indicated days. For reference method II[4], 2x10^{5} *SOX9*$^{+}$ scl-progenitors were seeded on MatriWS-coated plate suspended in 20µl base chondrogenic medium (DMEM supplemented with 1% v/v ITS, 50µg/ml ascorbic acid, 40µg/ml L-proline, 0.1µM DEX) supplemented with 20 ng/ml bFGF to adhere at 37℃ for 30min-1hr. The micromass was then covered by the base chondrogenic medium supplemented with TGFβ3 (10ng/ml) and was maintained in the medium for indicated days. For reference method III[5], the chondrospheroids were generated using *SOX9*$^{+}$ scl-progenitors by centrifugation as mentioned above. The spheroids were cultured in APEL2 (Stem cell technology, Cat#05270) supplemented with 5% PFHM II (ThermoFisher, Cat# 12040077), 20 ng/ml bFGF, and 1%

Pen-strep for indicated days. All differentiation was conducted in the same time frame (4 days) for comparison by RT-qPCR.

hBMSCs differentiation

Both the hBMSCs (human bone marrow mesenchymal stem cells) were dissociated into single cells using TrypLE and were differentiated into chondroprogenitors using the current protocol as mentioned above. The hBMSCs-derived chondroprogenitors were harvested on day 4 for analysis by RT-qPCR.

Articular cartilage repair assays

All animal experiments were approved by the Institutional Animal Care and Use Committee at the College of Life Sciences, Sichuan University. All animals were maintained under standardized conditions with the temperature- and light-controlled (12hr light/dark cycle), in individually ventilated cages always with companion mice, and had free access to food and water.

Male NOD/SCID mice (NOD/ShiLtJGpt-Prkdc^{em26Cd52}/Gpt, #T001492, GemPharmatech Co., Ltd, Jiangsu) at age of 8 weeks old were used for articular cartilage repair assay. The NOD/SCID mice were anesthetized with avertin (1.25% w/v 2,2,2-tribromoethanol) at 20μl/g (body weight).

The working solution of avertin was prepared by mixing 1.25g of 2,2,2-tribromoethanol (Sigma-Aldrich, Cat#T48402) with 2.5ml of 2-methyl-2-butanol (Sigma-Aldrich, Cat#721123) and dissolving in 97.5ml of UP H_2O. Subsequently, the skin of implantation sites was shaved and sterilized under standard procedures. A 3-mm incision was made medial to the left knee, and the femoral condyle was exposed by lateral dislocation of the patella. A 0.5-mm wide and 1-mm depth focal defect was then made on the articular surface of the femur to reach the underlying subchondral bone. Subsequently, 2~3 chondrospheroids were implanted in the defect site and secured by the blood from the subchondral area. After the patella was repositioned, the muscle and skin were closed with 8-0 and 6-0 prolene suture, respectively. The mice with defects but no cell implantation were prepared as sham controls.

Transplantation of *SOX9*$^+$ scl-progenitors under renal capsule

Day-4 *SOX9*$^+$ scl-progenitors were dissociated to single cells by TrypLE and 5.0x10^5 cells were suspended in CDMi supplemented with SAG (200μM), LDN193189 (600nM) and Y27632 (10μM) followed by centrifugation at 300 g for 6 mins in the U-shaped ultra-low attachment 96-well plate for spheroid formation overnight. Before transplantation, the spheroids were embedded in 10μl of Collagen I-Matrigel mixture (1:1) at 37℃ for 5 min for solidification. The Collagen I solution (2.4mg/ml) used

was prepared by adding 1ml of MEM (10x, Gibco, Cat# 11430030) to 8ml of Type I Collagen stock (3mg/ml, Bovine, Advanced Biomatrix, Cat# 5005). After pH adjustment to 7.0 by 1 M NaOH, the volume was brought to 10ml by DMEM (Gibco, Cat# 11965092).

For transplantation, 8-week NOD/SCID mice were anesthetized, and the skin was disinfected as mentioned above. The embedding with spheroids was transplanted under the kidney capsule, and the grafts were collected 8 weeks post-transplantation for histological evaluations.

Derivation of osteogenesis-committed early chondrocytes in micromass culture

6µl of MatriWS was first added on the central region of a 24-well plate for pre-coating at 37 ℃ for at least 1hr. $2x10^5$ *SOX9*$^+$ scl-progenitors resuspended in 5µl of CDMi containing SAG (200nM), LDN193189 (600nM), and Y27632 (10µM) were seeded on the coated region and were cultured for 30 min at 37℃ to adhere. Subsequently, an adequate volume of medium mentioned above was added to cover the cells. The next day, the medium was changed to the induction medium formulated as CDMi containing SAG (200nM), BMP2 (100ng/ml), and 1% v/v knockout serum replacement (KSR, Gibco, Cat#10828082) after washing with the wash

medium. The micromass was then cultured for 12 days with medium refreshed daily.

Subcutaneous injection of micromass-derived early chondrocytes

Each micromass was dissociated into single cells by Accutase and was suspended in 70μl of Matrigel. The skin was then tented using the tip of the insulin syringe (BD, Cat#324903), and the cells were injected into the subcutaneous site left to the midline of mice.

Histological section and evaluations of tissues

For the frozen section, all the samples were fixed with 4% PFA overnight and were dehydrated in 30% sucrose solution until sunk. The knee joints were decalcified in 19% w/v EDTA (in PBS, pH=7.0) at RT for 24 hours before dehydration. The dehydrated tissues were then embedded with OCT (optimal cutting temperature, SAKURA, Cat#4583) and placed at -80℃ for 24 hours. All the samples were sectioned at 6μm thickness with the microtome cryostat (Leica M1950). Tissue sections were stored at -20℃ before histological analysis.

For the paraffin section, the samples were fixed with 4% PFA overnight and were dehydrated in a serial gradient of ethanol from 50% to 100%. Also, the knee joints were decalcified before dehydration as mentioned

above. After tissue clearing in xylene, the samples were embedded in paraffin overnight.

For the histological staining, sections were re-hydrated and stained with H&E, Masson's trichrome (Solarbio, Cat# G1346), and Oil red (Solarbio, Cat# G1260) according to the manufacturer's protocol. Safranin O, Toluidine blue, and Alcian blue staining were conducted as mentioned before.

For the immunofluorescent staining. Antigen retrieval was conducted after re-hydration, if necessary, followed by a double rinse in PBS to remove the retrieval reagent. For intracellular antigens, the sections were permeabilized with 0.3% Triton X-100 for 8 min. Then, the sections were treated with blocking solution (5% BSA in PBS) at RT for 1hr and incubated overnight at 4°C with primary antibodies diluted in blocking solution. Secondary antibodies were stained at RT for 1hr protected from light and then washed three times with PBS before being mounted with DAPI. All the sections were examined under Leica TCS SP5II and Olympus VS200. Buffers for antigen retrieval are shown below:

Antibodies	Retrieval buffers	Conditions
Collagen II	Pepsin (Sigma-Aldrich, Cat#R2283)	37°C, 30 min

KU80 (Stem 101)	Antigen retrieval solution (Solarbio, Cat#C1035) only, frozen sections only	RT, 10 min
Collagen X	Citrate buffer (pH 6.0) or Tris-EDTA buffer (pH 9.0)	Boiled for 15 min
Collagen I	Antigen retrieval solution (Solarbio, Cat#C1035) or Citrate buffer (pH 6.0)	RT for 10 min or boiled for 15 min for citrate buffer
Endomucin (EMCN)	Antigen retrieval solution or Citrate buffer (pH 6.0)	RT, 10 min or boiled for 15 min
Leptin Receptor (LepR)	Citrate buffer (pH 6.0)	Boiled for 15 min
CXCL12	Citrate buffer (pH 6.0)	Boiled for 15 min
SCF	Citrate buffer (pH 6.0)	Boiled for 15 min
VEGF	Tris-EDTA buffer (pH 9.0)	Boiled for 15 min
COMP	Citrate buffer (pH 6.0) or Tris-EDTA buffer (pH 9.0)	Boiled for 15 min
SCD1	Antigen retrieval solution	RT, 10 min
CD45-biotin	Unnecessary	N.A.
Aggrecan (ACAN)	Tris-EDTA buffer (pH 9.0) for frozen or paraffin-embedded sections but unnecessary for immunocytochemistry (e.g., micromass culture)	Boiled for 15 min
Human Vimentin (VIM)	Unnecessary but all of the retrieval buffers worked and had no influence on staining results	Boiled for 15 min for heat-mediated antigen retrieval
PCNA	Unnecessary but also worked using the antigen retrieval solution	RT for 10 min if done
PRG4	Unnecessary	N.A.

Atomic force microscopy

Mice used in the articular cartilage repair assay were euthanized 4 weeks post-transplantation. Freshly harvested femoral condyles were fixed onto glass slides by tissue glue and covered with PBS to prevent dehydration. The samples were then measured by Brucker ICON Atomic Force

Microscope with pre-calibrated biosphere B50-NCH with silicon cantilever (spring constant k=40N/m, Poisson's ratio=0.5) (Nanotools, NT_B50_v0010). Analysis was performed with Nanoscope Analysis 2.0 Software.

Comparative evaluation assays for osteogenesis-committed early chondrocyte generation

Newly developed protocol vs the reference method

Osteogenesis-committed early chondrocyte induction using the newly established protocol has been described above. For the reference method[4], $2x10^5$ *SOX9*$^+$ scl-progenitors were seeded on MatriWS-coated plate in 20µl base chondrogenic medium (DMEM supplemented with 1% v/v ITS, 50µg/ml ascorbic acid, 40µg/ml L-proline, 0.1µM DEX) supplemented with 20 ng/ml bFGF to adhere at 37°C for 30min-1hr. The micromass was then covered by base chondrogenic medium supplemented with TGFβ3 (10ng/µl) only and was maintained in the medium for 10 days at which time the TGFβ3 was replaced by 50ng/ml BMP4 for the rest of the experiments (2 days). All the differentiation was conducted in the same time frame (12 days) for comparison by RT-qPCR.

hBMSCs differentiation by the newly developed protocol

The hBMSCs were differentiated by micromass using the newly developed protocol following the same cell seeding procedure. The medium was refreshed every day. The samples were harvested on day 12 for analysis by RT-qPCR.

Characterization of skeletal stem cells in micromass-derived tissues.

The micromass was generated from *SOX9*$^{+}$ scl-progenitors as mentioned above followed by subcutaneous injection to NOD/SCID mice. The grafts were collected 8 weeks post-transplantation and were dissociated to single cells by Collagenase I and Collagenase II mixture and were stained by indicated reagent and antibodies (DAPI, CD235a, CD31, TIE2, CD45, PDPN, CD146, CD73, CD164). The gating strategy was carefully defined using Fluorescence Minus One (FMO) control.

Tissue clearing for the imaging of immunostained micromass.

The osteogenesis-committed early chondrocytes were generated using *SOX9*$^{+}$ scl-progenitor on the confocal dish as mentioned above, and the immunostaining was conducted with routine protocols. After nuclear staining by Hoechst 33342, the cells were dehydrated in the increasing gradient of 30%, 50%, 80%, and 100% ethanol for 5 min each. After an extra 10 min dehydration in 100% ethanol, the cells were cleared with a clearing solution containing 80% v/v ethyl cinnamate (ECi) (Sigma, Cat#

112372) and 20% v/v polyethylene glycol (Sigma, Cat# 447943) at RT for 10 min. For longer preservation, the samples were immersed in the clearing solution at 4℃ until imaging with the lid sealed by paraffin film. The imaging was conducted with Olympus SpinSR10.

Construction of osteochondral fusions

The osteochondral fusions were constructed by the integration of chondroprogenitors and osteogenesis-committed early chondrocytes. The chondroprogenitors were first generated by pelleting $1x10^5$ *SOX9*$^+$ scl-progenitors suspended in CI medium supplemented with Y27632 (10μM) in Ultra-low attachment 96-well plate and were cultured in CI medium for 14 days for chondrogenic differentiation and maturation. The osteogenesis-committed early chondrocytes were prepared as mentioned above in micromass (12 days). For reconstitution, the osteogenesis-committed early chondrocytes were first dissociated to single cells by Accutase and resuspended using CI medium supplemented with 10μM of Y27632 as 70μl/micromass. Each micromass suspension (70μl) was then placed into the low attachment plate for another round of centrifugation at 300g for 6 min. The day-14 chondroprogenitors were then fused with the pelleted osteogenesis-committed early chondrocytes at 1:1 by centrifugation at 300g for 6 min. After being cultured overnight, the medium was changed to CI medium without Y27632. The fusions were cultured *in vitro* for 7

days in CI medium with the medium changed every 2 days before transplanted under the renal capsule of NOD/SCID mice. The osteogenesis-committed early chondrocyte alone (osteo-spheroids) and chondroprogenitor alone (chondro-spheroids), were prepared as control and were cultured in the CI medium for the same duration (7 days) followed by transplantation.

Bulk-population RNA sequencing for cell-surface marker screening

For bulk-population RNA-seq analysis, day-0 hPSCs were differentiated to sclerotome using the traditional 6-day protocol (Figure S2A), and total RNAs were extracted respectively from undifferentiated human PSCs, sorted *SOX9*$^{+}$ scl-progenitors and *SOX9*$^{-}$ cells using TRI Reagent. Library preparation and sequencing were performed by Novogene Inc., Beijing. Raw data in FASTQ format were obtained using the CASAVA 1.8.4 pipeline and were filtered to remove the adaptors and paired reads with N.A. bases of more than 10%. The clean reads were then mapped to hg19 using the HISAT2 software (version 2.2.1). Read counts for each gene were based on sense-strand data obtained using the featureCounts (version 2.0.1) software from the Subread package. Transcripts per million (TPM) was used for the normalization of gene expression levels accounting for both gene length and sample variations[6]. Differential gene expression analysis was conducted using edgeR[7] comparing the *SOX9*$^{+}$ scl-progenitors

vs *SOX9*$^{-}$ cells and *SOX9*$^{+}$ scl-progenitors vs hPSCs. For cell-surface marker screening, the candidate list was obtained by setting the cutoff line as $\log_2$(fold change)>1 and adjusted *p*-value<0.05. Genes with TPM<50 in the *SOX9*$^{+}$ population and genes with TPM>50 in *SOX9*$^{-}$ cells were excluded. Moreover, genes with TPM>10 in hPSCs were further discarded to remove any genes enriched in undifferentiated cells. Bulk-population RNA-seq data have been submitted to the GEO database (GSE222109).

Single-cell RNA sequencing by cell hashing

Cell hashing using TotalSeqTM reagents enabled to run simultaneously multiple samples in a single lane. Undifferentiated PSC, PS, PSM, and *SOX9*$^{+}$ scl-progenitors derived by the new method (Figure 1A) were prepared as mentioned above and were dissociated into single cells using Accutase. After being washed once with 5% BSA (in PBS), the cells were filtered with a 40-μm strainer followed by cell counting. Then indicated number of cells was incubated with TotalSeq^{TM} antibodies using the manufacturer's protocol. The antibody information is shown below:

Antibody names	Cat# (BioLegend)	Targeting cells
TotalSeq™-A0251 anti-human Hashtag 1	394601	hPSC
TotalSeq™-A0252 anti-human Hashtag 2	394603	PS
TotalSeq™-A0253 anti-human Hashtag 3	394605	PSM
TotalSeq™-A0254 anti-human Hashtag 4	394607	*SOX9*$^{+}$ scl-progenitors

After washing and resuspending in 0.4% BSA (in PBS), an equal number of cells from each stage were combined for cell viability assessment using Countess II Automated Cell Counter (Thermo Fisher, Cat# AMQAX1000). Library preparation and sequencing were performed by Singleron Biotechnologies Inc, Nanjing using GEXSCOPE® Single Cell RNA Library Kit for library construction. Key information including single-cell processing, capture, cDNA purification, amplification, library construction, and library purification is shown below：

Parameters	Values
Load cell count	23,000
Suspension concentration (cells/μl)	230
Cell viability	81%
PCR cycles	13
Library concentration (ng/μl)	1.52 (HTO library); 18.2 (mRNA library)
Library size	244 (HTO library *); 498 (mRNA library)
Sample index	D701

* Note: the read 1 sequence on beads from GEXSCOPE® Single Cell RNA Library Kit was ~120bp, resulting the HTO library size around ~230 bp rather than ~180 bp for 10x Genomics single index kits.

Processing of scRNA-seq raw data

The CeleScope software was obtained from Git Hub (https://github.com/singleron-RD/CeleScope). Alignment, filtering, barcode counting, and UMI counting were performed with the CeleScope pipeline to generate a feature-barcode matrix and determine clusters. scRNA-seq data has been submitted to the GEO database (GSE232109).

t-SNE visualization of cell clustering, reconstruction of differentiation trajectories, and gene expression dynamics profiling

The feature-barcode matrix was processed using R and Seurat v4.2.0 package. For the hashtag library, signal-to-noise ratio (SNR) was defined as the ratio of the highest and the second-highest UMI count value of hashtags for a certain cell. Cells that have low UMI counts (highest UMI count less than 200) and low SNR (SNR less than 20) were filtered out as multiplets and undetermined cells. The cells with mitochondrial gene content of more than 10% were further removed as low-quality cells. Subsequently, we filtered out cells that have UMI counts (number of counts，nCounts) and expressing genes (number of features, nFeatures) under the 10_{th} percentile among the total cells. The gene expression levels for each cell were normalized by the sequencing depth, multiplying a default size factor of 10,000, and then log-transformed. To reduce variance introduced by cell cycle stages, Seurat's function "ScaleData" was used to regress out the effect with cell cycle markers extracted from the Seurat database

"cc.genes"[8]. Next, dimensionality reduction and visualization were performed using Seurat's function "RunTSNE" where the cells were colored by hashtags in a tSNE (t-Distributed Stochastic Neighbor Embedding) plot.

For reconstruction of differentiation trajectories, datasets of cells passed the quality control steps including UMI and SNR threshold for hashtag library, mitochondrial gene filtering, nCount, and nFeature threshold as mentioned above were converted into a Monocle object using Monocle 2. To cluster and order the cells in an unsupervised approach, the genes used were selected based on the level of expression and variability across cells by using the "dispersionTable" function to calculate the average expression and dispersion values of each gene. Genes with a mean expression higher than 1 were selected and the trajectories were visualized using the Monocle function "plot_cell_trajectory". Subsequently, the gene expression dynamics across the trajectories were plotted using the "plot_genes_in_pseudotime" function to show the correct induction of stage-specific genes during differentiation.

Re-clustering of mouse organogenesis atlas

To test if *Itga9* was expressed in primary sclerotome during mouse development, single-cell RNA sequencing data on mouse embryonic

organogenesis was re-analyzed by dissecting the sclerotome populations present in the E10.5 embryo from the reference atlas[9] and re-clustered using non-linear dimensionality reduction algorithm to visualize clusters on T-distributed Stochastic Neighbor Embedding (t-SNE) plot. The sclerotome population was annotated using differentially expressed genes.

Expansion culture and cryopreservation of *SOX9*$^+$ scl-progenitors

SOX9$^+$ scl-progenitors were dissociated to single cells with Accutase. After being washed once with wash medium, cells were seeded on MatriWS-coated plates at 1x10^5 cells/cm^2 and cultured with CDMi supplemented with SAG (200nM), LDN19389 (600nM), Y27632 (10μM), bFGF (10ng/ml), 1% v/v KSR, 5mg/ml BSA for 3~5 days before the next splitting. The medium was refreshed every day. For cryopreservation of *SOX9*$^+$ scl-progenitors, 1x10^6 dissociated cells were resuspended with 1ml CryoSO free (Sigma-Aldrich, Cat#C9249-100ML) with Y27632 (10μM) and preserved in liquid nitrogen after being placed in isopropanol-containing chamber overnight at -80℃. Upon thawing, the cells were seeded on 24-well cell culture plates for recovery.

Bioluminescence imaging

AkaLuc-BsdR UC01 (*SOX9-IRES-tdTomato*) hPSCs was constructed by co-transfection of piggyBac transposase-expressing vector (pBase) and the

donor vector harboring *EF1-AkaLuc* and *PGK-EM7-BsdR* cassette between transposon-specific inverted terminal repeats (ITR) to the parental UC01 (*SOX9-IRES-tdTomato*) cells. Articular cartilage repair assays were conducted using the *AkaLuc-BsdR* cell-derived chondroprogenitors. Upon imaging, the mice were injected with 10mM AkaLumine-HCl (MeisenCTCC, Cat#CTCC-LUC-002) intraperitoneally 15min before imaging and were anesthetized with avertin 5min before imaging and were then placed in the IVIS Spectrum imaging chamber. Images were taken every two weeks from week 2 to week 8 with constant settings. Analysis was conducted with Living Image software.

Statistical analysis

Statistical analysis was performed with SPSS 26.0 software. All data were expressed as mean ± standard deviation (SD). The statistical method used for each dataset was described in the figure legend respectively. $p<0.05$ was deemed statistically significant.

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