

Stem Cell Reports, Volume 19

Supplemental Information

MSX1⁺PDGFRA^{low} limb mesenchyme-like cells as an efficient stem cell source for human cartilage regeneration

Yuansong Liao, Fanchen Kang, Jingfei Xiong, Kun Xie, Mingxu Li, Ling Yu, Yuqing Wang, Hanyi Chen, Guogen Ye, Yike Yin, Weihua Guo, Haoyang Cai, Qing Zhu, and Zhonghan Li

Supplemental Figures

Figure S1

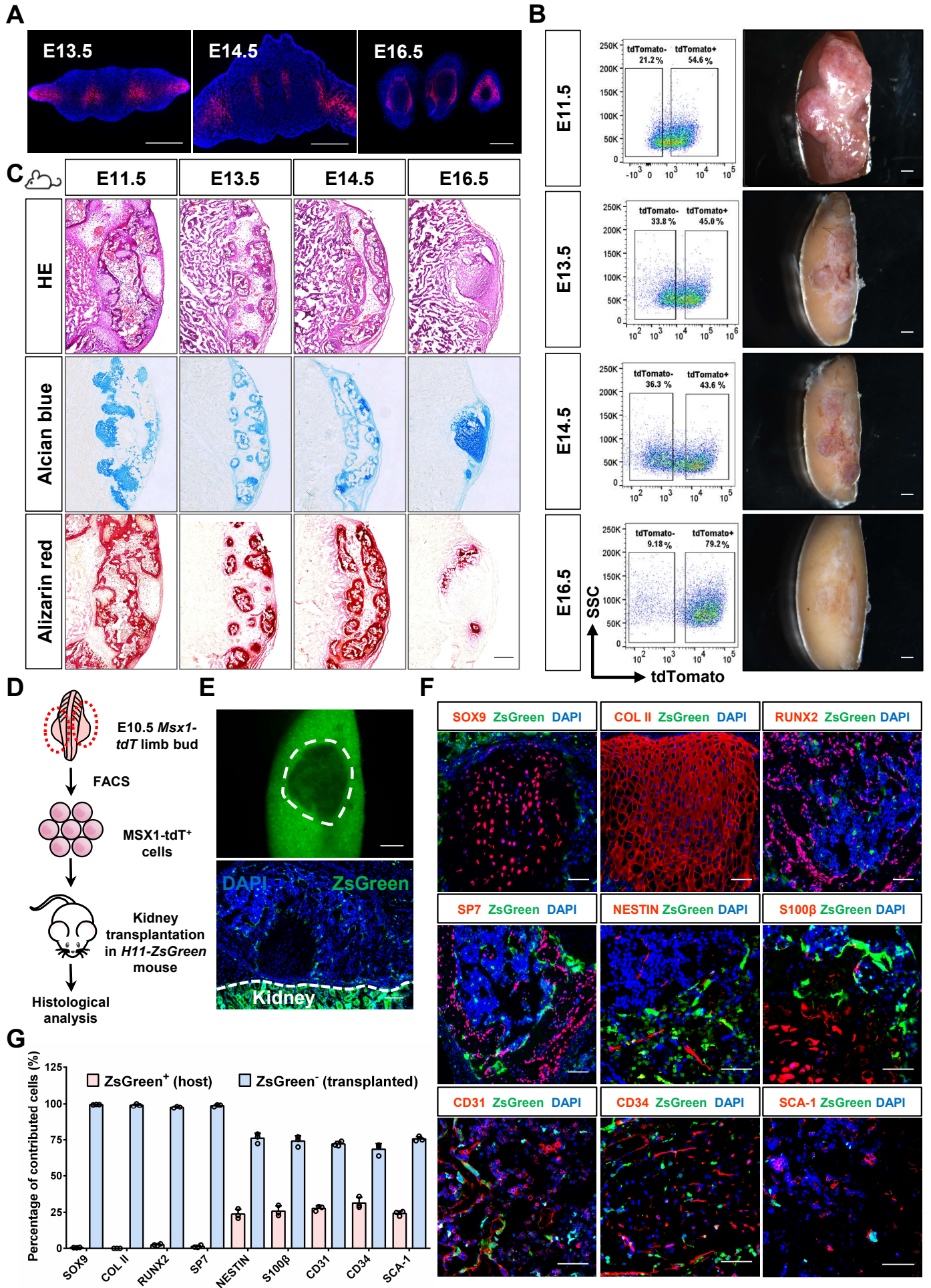
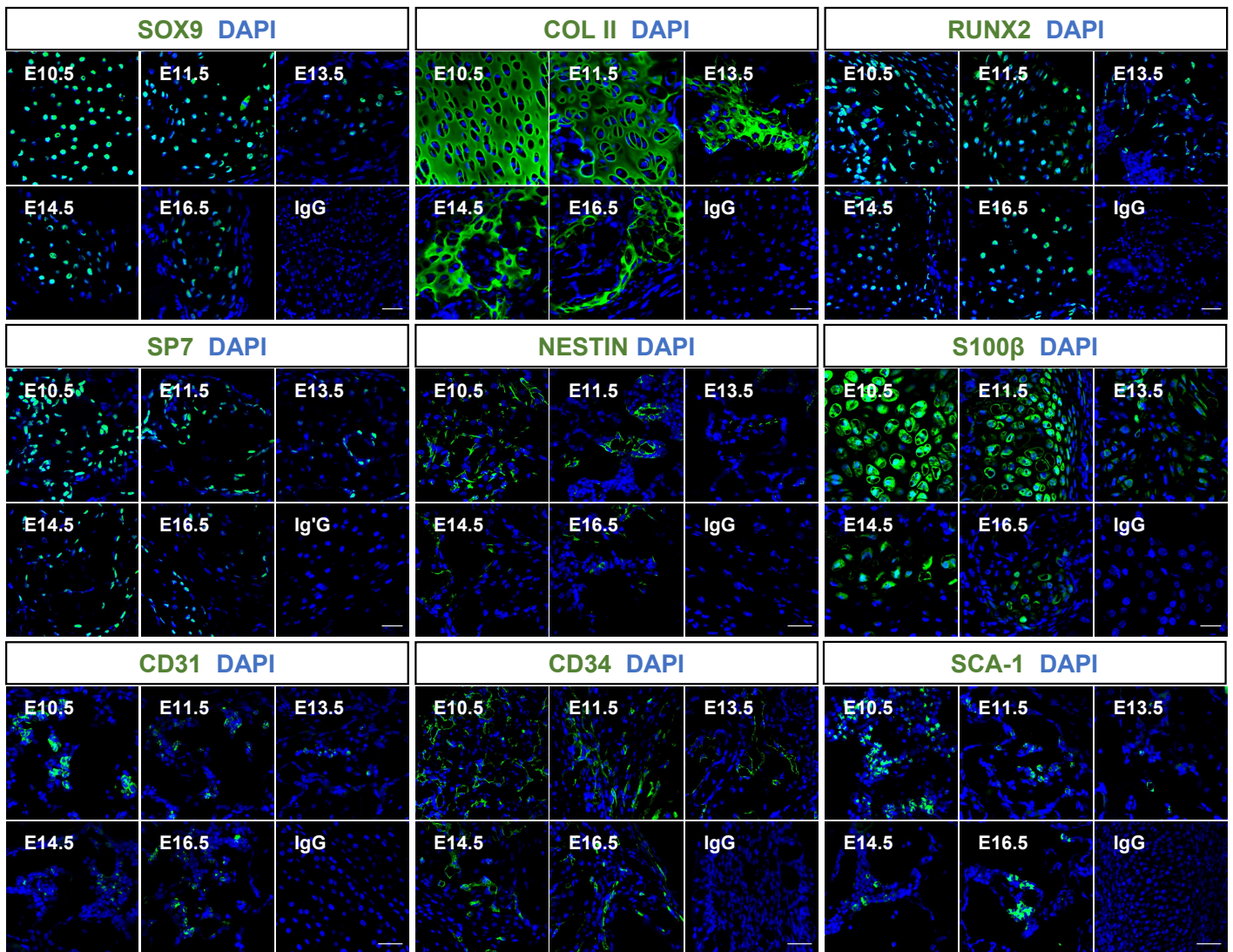


Figure S1. Primary limb bud-derived MSX1⁺ mesenchymal progenitors exhibited strong bone regenerative capability. (A) Longitudinal sections of the hind limb at E13.5/E14.5/E16.5 stages. The expression of MSX1 was located in the interdigital regions. Scale bars: 200 μ m. (B) MSX1⁺ cells isolated from the selected stages could form bone-like tissues under the kidney capsule. Left: isolation of primary MSX1⁺ progenitors by cell sorting; Right: representative images of the bone-like tissues formed by the sorted MSX1⁺ progenitors from the corresponding stages. Scale bars: 1 mm. (C) Histology analysis of bone-like tissues formed by E11.5, E13.5, E14.5, and E16.5 MSX1⁺ cells. Representative images of HE, Alcian blue, and alizarin red staining were shown. Scale bars: 250 μ m. (D) Schematic illustration of MSX1⁺ cell transplantation in the ZsGreen recipient mice. MSX1⁺ cells were transplanted in a ZsGreen-expressing host to verify the cell origin of regenerated bone-like tissues. (E) Immunofluorescence images showing bone-like tissues were ZsGreen negative. Scale bars: 1 mm (top), 100 μ m (bottom). (F-G) Immunostaining and quantitative analysis of different cell lineages within the transplants. Almost all chondrocytes, osteocytes, and most of the neural and VECs were derived from the transplanted limb MSX1⁺ mesenchymal progenitors. Scale bars: 50 μ m. Error bars represented data from three samples of three independent experiments.

Figure S2

A



B

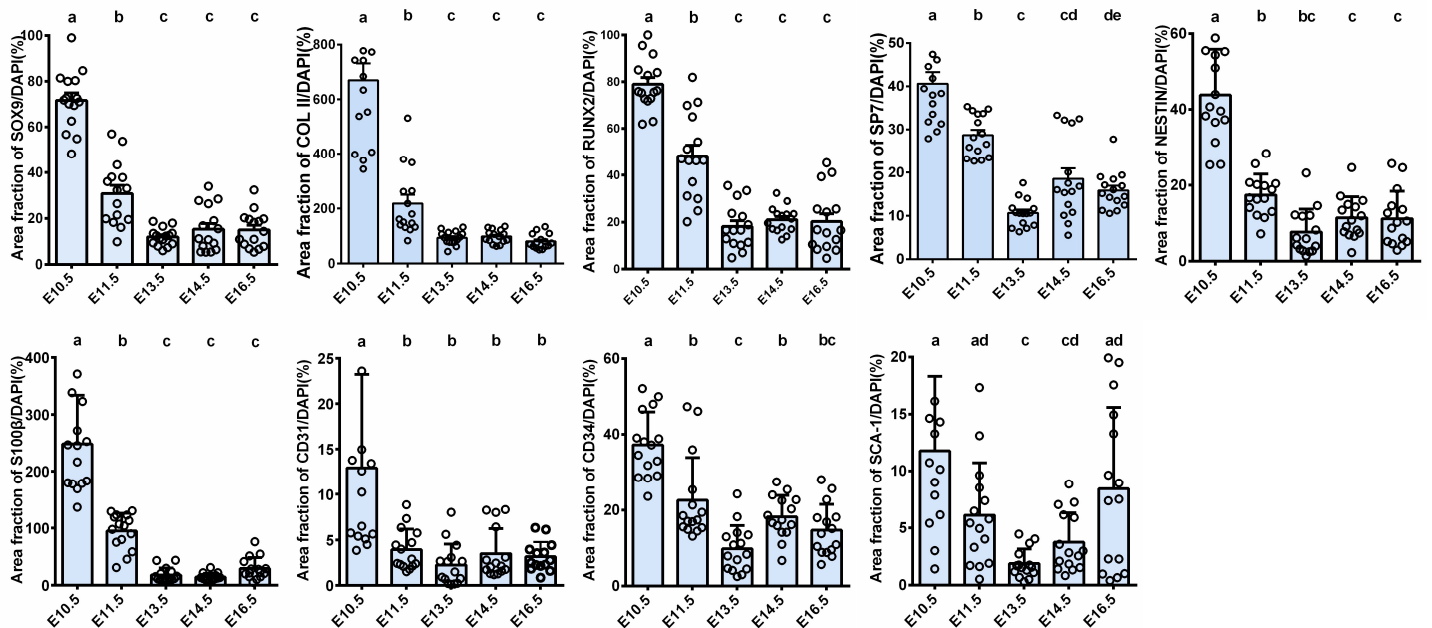


Figure S2. E10.5 limb bud MSX1⁺ mesenchymal progenitors exhibited strong bone regenerative capability. (A) Immunostaining characterization of osteochondral markers in the regenerated bone-like tissues using MSX1⁺ cells from different developmental stages. The markers were the same as in Figure 1F. Scale bars: 25 μ m (SOX9, COL II, RUNX2, and SP7) and 30 μ m (NESTIN, S100 β , CD31, CD34, and SCA-1). (B) Quantitative analysis of osteochondral markers in regenerated bone-like tissues. The results confirmed that E10.5 MSX1⁺ cells had the most efficient regenerative potential. Error bars represented data from fifteen sections of three independent transplantation experiments (with at least three transplants for each group, mean \pm SD). Statistical significance was marked as different letters. Different letters between the two groups indicated statistical significance while possessing the same letter indicated otherwise. Statistics: One-way ANOVA followed by Tukey (CD34) and Tamhane's T2 (SOX9, COL II, RUNX2, SP7, NESTIN, S100 β , CD31, and SCA-1) post hoc multiple comparisons by SPSS v22.0.

Figure S3

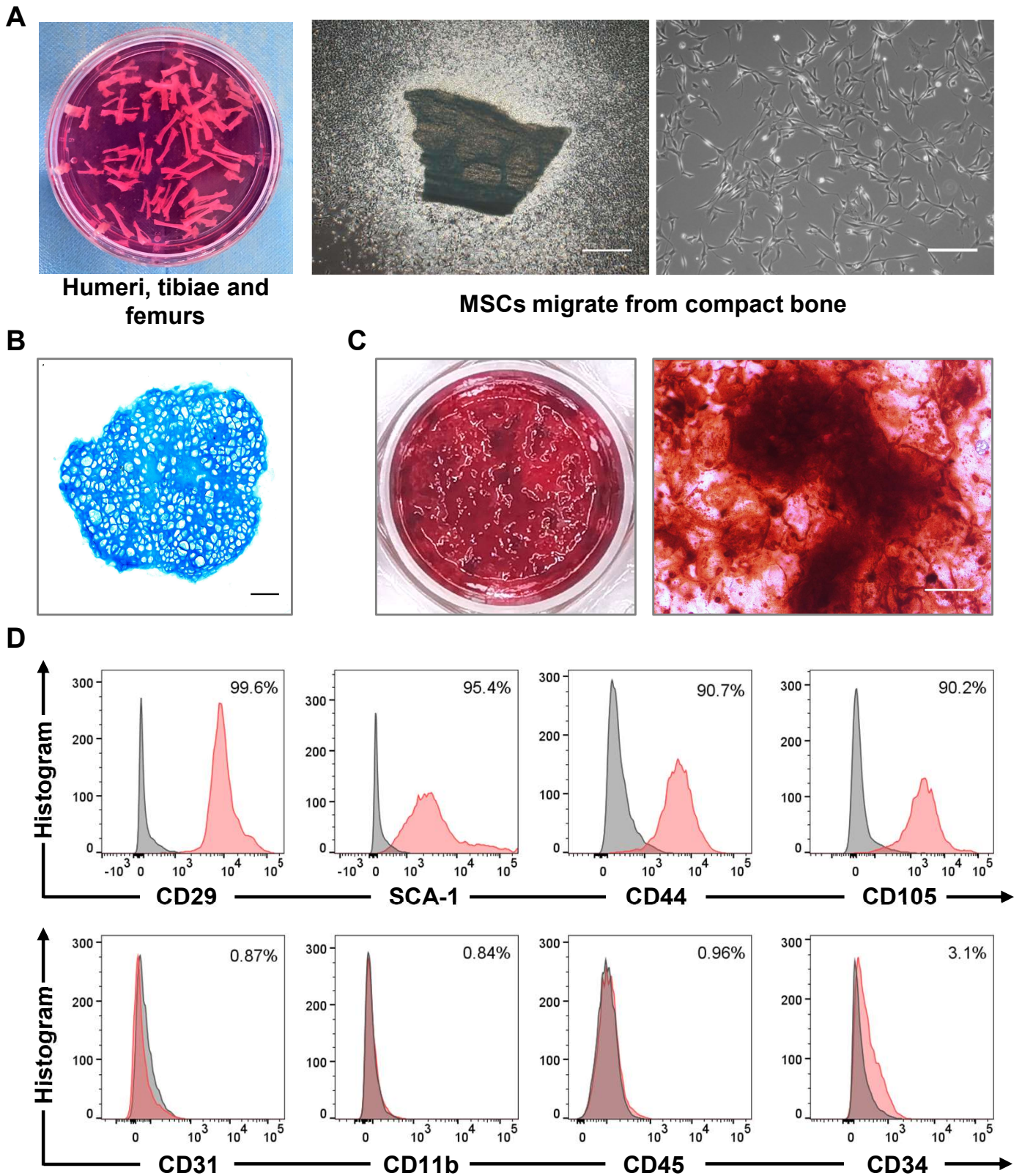


Figure S3. The isolation and characterization of MSCs from murine compact bones. (A) Isolation and culture of MSCs from the compact bones. Left: isolated mouse compact bones. Middle: MSCs migrated out from the bone chips. Right: morphology of isolated MSCs. Scale bars: 500 μm (middle), 100 μm (right). (B-C) Multilineage differentiation of the isolated MSCs. Chondrogenesis was evaluated by Alcian blue (left) and osteogenesis by alizarin red staining (right). Scale bars: 50 μm (left), 100 μm (right). (D) Flow cytometry analysis confirmed the presence of classical MSC markers in the isolated cells. Isolated MSC cells were positive for SCA-1, CD29, CD44, and CD105, but negative for CD11b, CD31, CD34, and CD45.

Figure S4

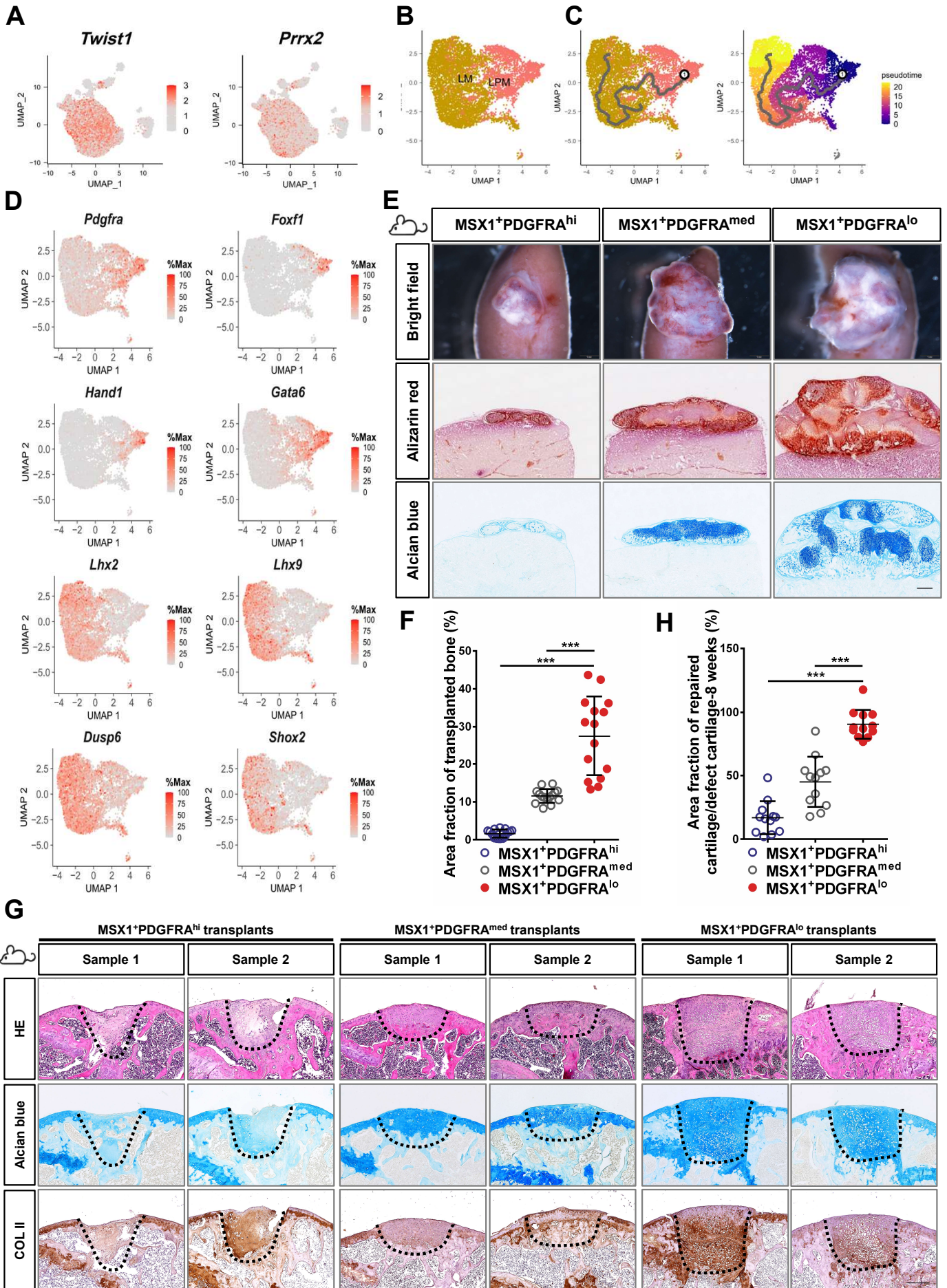


Figure S4. ScRNA-seq analysis identified MSX1⁺PDGFRA^{low} limb bud cells as the key cell composition with strong bone regenerative capability. (A) Most of the E10.5 MSX1⁺ progenitor cells highly expressed mesenchymal makers *Prrx2* and *Twist1*. (B) UMAP plots of mesenchymal cells exhibited the two major subsets (LPM and LM cells) for downstream trajectory analysis. (C) Pseudotime trajectory analysis identified the LM cells as the descendants of LPM. (D) UMAP plots showing the marker gene expressions for LPM cells (*Pdgfra*⁺ *Foxf1*⁺ *Hand1*⁺ *Gata6*⁺), LM cells (*Lhx2*⁺ *Lhx9*⁺ *Dusp6*⁺ *Shox2*⁺). (E) Confirmation of the bone regeneration capability of MSX1⁺PDGFRA^{low} cells *in vivo*. The sorted cells were transplanted into the kidney capsule of the recipient mice and analyzed after three weeks. Representative images were shown. Alcian blue staining was used to detect cartilage formation and alizarin red staining for osteogenesis. Scale bars: 1 mm (BF), 500 μ m (Alcian blue and alizarin red). (F) Quantitative analysis of bone-like tissues indicated that the MSX1⁺PDGFRA^{low} cells retained the strongest osteochondral competency among the tested cell populations. Error bars represented data from fifteen sections of three independent experiments with at least three transplants for each group (mean \pm SD). Statistics: One-way ANOVA followed by Tamhane's T2 post hoc multiple comparisons by SPSS v22.0. *** $p < 0.001$. (G) Regenerated cartilage by mouse MSX1⁺PDGFRA^{low} cells could be maintained for at least 8 weeks. Samples were analyzed 8 weeks post-transplantation. The defect sites were filled with hyaline chondrocytes confirmed by Alcian blue and COL II staining. Scale bars: 200 μ m. (H) Quantitative analysis revealed MSX1⁺PDGFRA^{low} cells possessed the highest cartilage regeneration efficiency. Error bars represented data from twelve sections of six mice in three independent experiments (mean \pm SD). Statistics: One-way ANOVA followed by Tukey post hoc multiple comparisons by SPSS v22.0. *** $p < 0.001$.

Figure S5

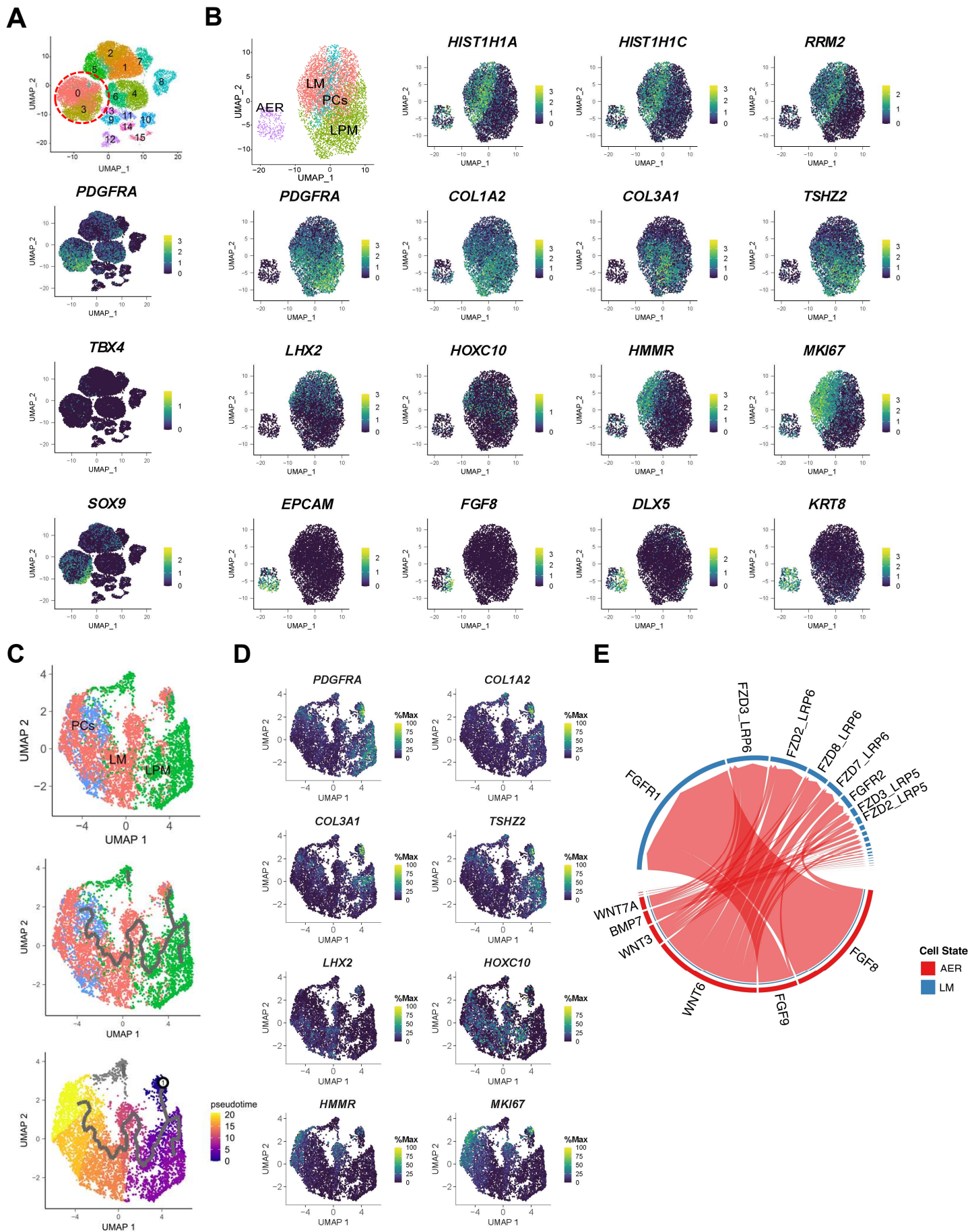


Figure S5. ScRNA-seq analysis of 5 WPC human limbs. (A) UMAP plots of gene expression profiles for primary human limb bud cells at 5 WPC identified 16 cell clusters. Based on the expression of *PDGFRA*, *TBX4*, and *SOX9*, clusters 0 and 3 were considered to be hindlimb cells (red dashed line). (B) Together with the identified hindlimb cells, human AER (cluster 10 in Figure S5A) was also isolated for downstream Cellchat analysis. UMAP plots of clusters 0, 3, and 10 were colored by cell type, including LM (red), LPM (green), PCs (proliferating cells of LPM and LM, blue), and AER (purple). The expressions of different marker genes in selected clusters were listed. PCs: *HIST1H1A*, *HIST1H1C*, *RRM2*. LPM: *PDGFRA*, *COL1A2*, *COL3A1*, *TSHZ2*. LM: *LHX2*, *HOXC10*, *HMMR*, *MKI67*. AER: *EPCAM*, *FGF8*, *DLX5*, *KRT8*. (C) Pseudotime differentiation trajectory analysis projected that human hindlimb cells sequentially undergo differentiation from LPM to LM. PCs: proliferating cells. (D) UMAP plots showing the expression of specific marker genes along the trajectory from human LPM (*PDGFRA*⁺ *COL1A2*⁺ *COL3A1*⁺ *TSHZ2*⁺), to human LM cells (*LHX2*⁺ *HOXC10*⁺ *HMMR*⁺ *MKI67*⁺). (E) Analysis of potential ligands and receptors by Cellchat to drive LM development. The results indicated that most of the candidates were enriched in WNT and FGF signaling pathways.

Figure S6

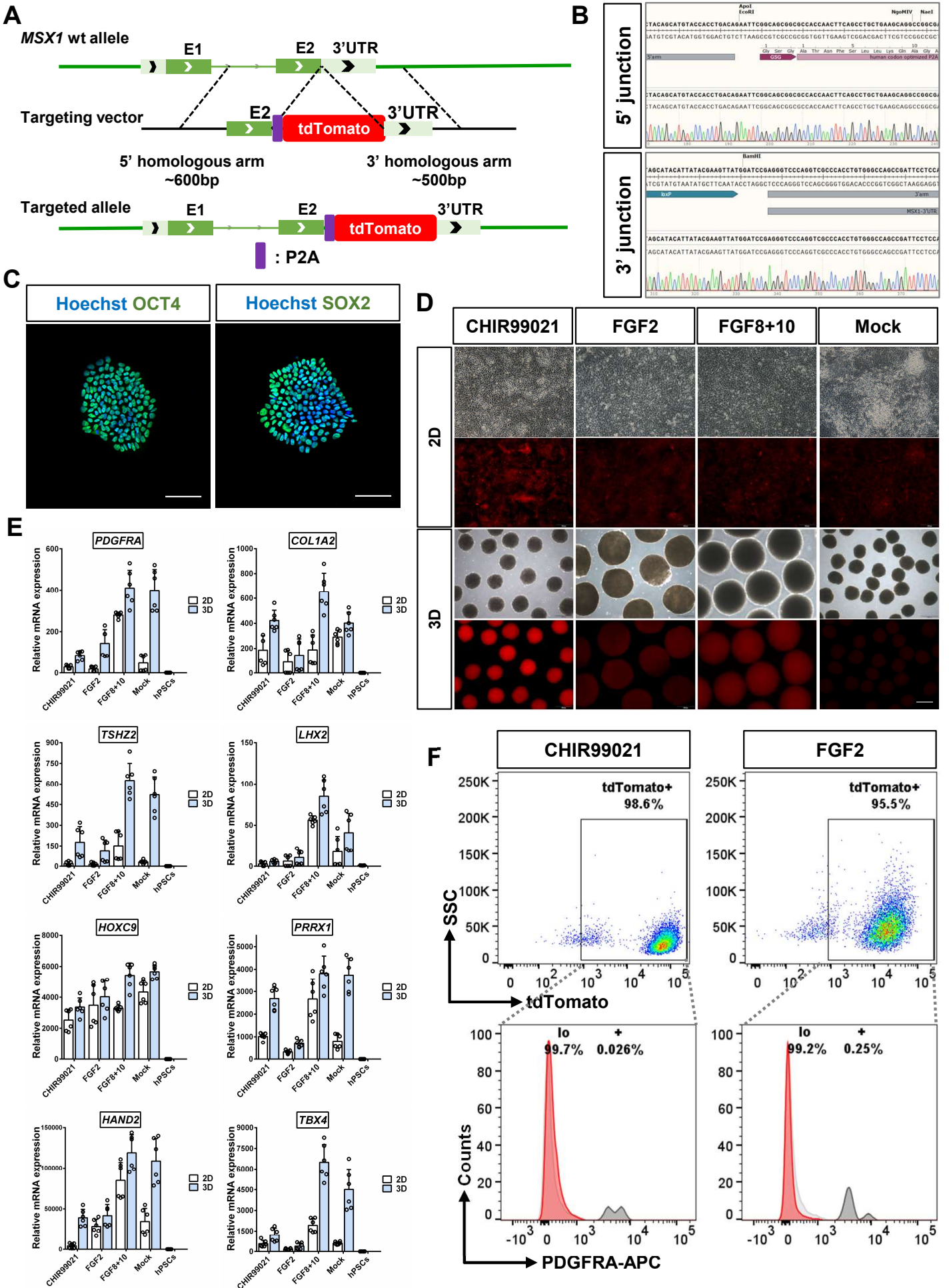


Figure S6. Generation of the *MSX1*^{P2A-tdTomato} knock-in cell line induction of LM-like cells in hPSCs. (A) Schematic procedure of cell line construction. A P2A-tdTomato cassette was inserted into the human *MSX1* loci by homologous recombination. (B) The P2A-tdTomato cassette was inserted into the loci behind exon2 of human *MSX1* by the HDR-based CRISPR/Cas9 method, confirmed by genome sequencing. (C) Immunostaining of SOX2 and OCT4 confirmed pluripotency of the knock-in cells. Scale bars: 75 μm . (D) Bright-field and fluorescent microscopy of day 8 *MSX1*⁺ (tdTomato⁺) cells following different treatments with small molecules and growth factors under 2D and 3D conditions. Scale bars: 500 μm . (E) qRT-PCR analysis revealed LM marker gene expressions were significantly enriched in FGF8+10 treated cells, especially in the 3D culture. LPM makers: *PDGFRA*, *COL1A2* and *TSHZ2*. LM makers: *LHX2* and *HOXC9*. Mesenchymal markers: *HAND2* and *PRRX1*. Hindlimb-specific marker: *TBX4*. Error bars represented data from two independent experiments with duplicates. Each sample contained three technique replicates. (F) FACS sorting of the CHIR99021 and FGF2 treated groups by tdTomato (*MSX1*) and *PDGFRA*.

Supplemental Tables

Table S1. List of primary antibodies used for immunostaining, related to Supplemental experimental procedures

Target	Antibody Name	Vendor	Catalog Number
Mouse CD29/ CD105/CD44/CD45/ SCA-1/CD11b	Anti-CD29/ CD105/CD44/CD45/ SCA-1/CD11b antibodies	R&D systems	SC018
Mouse CD31	Anti-CD31 antibody	Invitrogen	11-0311-82
Mouse CD34	Anti-CD34 antibody	Invitrogen	11-5981-82
Mouse PDGFRA	anti-PDGFRA antibody	Biolegend	135907
Mouse SOX9	anti-SOX9 antibody	HUABIO	ET1611-56
Mouse COL II	anti-COL II antibody	Invitrogen	MA5-12789
Mouse SP7	Anti-SP7 antibody	Abcam	ab209484
Mouse RUNX2	anti-RUNX2 antibody	HUABIO	ET1612-47
Mouse NEATIN	anti-NESTIN antibody	HUABIO	R1510-19
Mouse S100 β	anti-S100 β antibody	HUABIO	ET1610-3
Mouse CD34	anti-CD34 antibody	HUABIO	ET606-11
Mouse SCA-1	anti-SCA-1 antibody	Invitrogen	11-5981-82
Human OCT4	anti-OCT4 antibody	Abcam	ab19857
Human SOX2	anti-SOX2 antibody	Abcam	ab97959
Human PDGFRA	anti-PDGFRA antibody	Biolegend	323512

Table S2. Primers used in genome sequencing of Msx1^{P2A-tdTomato} knock-in hPSCs cell line, related to Result and Experimental procedures sections.

Targets	Direction	Sequence (5'-3')
Msx1-5'	Forward	CACATCTTCCCAGCTGTTTAGGCC
Msx1-3'	Reverse	CCTACCTTTGCAACACATCTGTGTTTTCC
tdTomato-1	Reverse	GAACTCTTTGATGACGGCCATGTTG
tdTomato-2	Forward	CCTCCGAGGACAACAACATGGCC
PGK-1	Reverse	CCATTTGTCACGTCCTGCACGAC
PGK-2	Forward	GCCTCGCACACATTCCACATCCA
Puro-1	Reverse	GGCTTGCGGGTCATGCACCA
Puro-2	Forward	CTTCTACGAGCGGCTCGGCTTCA

Table S3. Primers of qRT-PCR used in this article were listed, related to Result and Experimental procedures sections.

Targets	Direction	Sequence (5'-3')
<i>GAPDH</i>	Forward	TGCCAAATATGATGACATCAAGAA
	Reverse	GGAGTGGGTGTCGCTGTTG
<i>PDGFRA</i>	Forward	GATTGTGGTCACCTGTGCTG
	Reverse	TCTTCCAGCATTGTGATGCC
<i>COL1A2</i>	Forward	GAGGACCACGTGGAGAAAGG
	Reverse	CAAAGTTCCCACCGAGACCA
<i>COL3A1</i>	Forward	GCTACTTCTCGCTCTGCTTC
	Reverse	CCGCATAGGACTGACCAAGA
<i>TSHZ2</i>	Forward	CCACCCACATGATGGTCACA
	Reverse	GCTTGGGAGCCAGAGAATCA
<i>LHX2</i>	Forward	GGACGGTAGCATCTACTGCAA
	Reverse	CCCGTGGTCAGCATCTTGTT
<i>HOXC10</i>	Forward	AAAGGAGAGGGCCAAAGCTG
	Reverse	TTCCTTCCGCTCTTTGCTGT
<i>HMMR</i>	Forward	GGCTGGTCAAGCAATTGGAA
	Reverse	CCTGGGTATGAGCAGCACTA
<i>MKI67</i>	Forward	ACACTCCACCTGTCCTGAAG
	Reverse	CCAAGCTTTGTGCCTTCACT
<i>HOXC9</i>	Forward	GCAGCAAGCACAAAGAGGAG
	Reverse	CAGCGTCTGGTACTTGGTGT
<i>TBX4</i>	Forward	TCCAGAAGCTGAAGCTGACA
	Reverse	GGAGCCGAAAGCATTGTTCT
<i>Gapdh</i>	Forward	GCACAGTCAAGGCCGAGAAT
	Reverse	GCCTTCTCCATGGTGGTGAA
<i>Pdgfra</i>	Forward	AGGCAGGGCTTCAACGGAAC
	Reverse	AAGACGGCACAGGTCACCAC
<i>Foxf1</i>	Forward	CTGGAGCAGCCATACCTTCA
	Reverse	TGAGTGATACCGAGGGATGC
<i>Hand1</i>	Forward	TGGCCAAGGATGCACAAGCA
	Reverse	AAGCTTTCGGGCTGCTGAGG
<i>Isl1</i>	Forward	TGGAGACCCTCTCAGTCCCT
	Reverse	AGCTGCTTCTCGTTGAGCAC
<i>Lhx2</i>	Forward	CTTCAGCAAGGATGGCAGCA
	Reverse	GCGCATCACCATCTCTGAGG
<i>Lhx9</i>	Forward	CCAGCTCTGGGAGTGGACAT
	Reverse	CCAAGTGGTCTGCCTCGTTC

Supplemental experimental procedures

Mouse strains and animal care

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 (25 °C, 12 h light/dark cycle), in individually ventilated cages, and had free access to food and water. Knock-in C57BL6-*Msx1*^{P2A-tdTomato} mice and *H11-ZsGreen* mice were custom-generated by Biocytogen, Inc. (Beijing). Mouse offspring from these strains were routinely genotyped using standard PCR protocols. C57BL6 wt mice and NOD-SCID mice were purchased from Gempharmatech Co., Ltd (Chengdu). C57BL6 wt mice were used as recipients for renal subcapsular and articular cartilage transplantation of mouse MSX1⁺ cells, while NOD-SCID mice were used as recipients for articular cartilage transplantation of differentiated human LPM- and LM-like cells.

Flow cytometry

Primary mouse MSX1⁺ (tdTomato⁺) cells were sorted by BD FACS Aria II (BD Biosciences, USA). To identify the surface markers, compact bone derived-MSCs were stained with anti-CD29/CD105/CD44/CD45/SCA-1/CD11b (R&D systems, Cat# SC018), CD31 (Invitrogen, Cat# 11-0311-

82), CD34 (Invitrogen, Cat# 11-5981-82) antibodies at 4 °C for 30 min. After washing in phosphate-buffered saline (PBS) (3 × 5 min), secondary antibodies were incubated for 30 min at room temperature (except for CD31 and CD34 staining). These cells were suspended in FACS sorting buffer after washing them in PBS (3 × 5 min).

Dissociation of mouse hindlimb cells and the differentiated human day 8 cells was performed with 0.25% Trypsin-EDTA (ThermoFisher, Cat# 25200072) at 37 °C for 5 min. Then digestion was terminated by adding a complete medium (DMEM + 15% FBS (Gibco, Cat# 10099-141) + 1% Glutamax + 1% NEAA + 0.1% β-mercaptoethanol + 1% Pen-Strep (Gibco, Cat# 15140122)). After centrifuged at 450 g for 3 min and resuspended in PBS, the collected cells were stained with anti-PDGFRα (Biolegend, Cat# 135907 (for mouse cells); Cat# 323512 (for human cells), 1: 100) antibodies on ice and then prepared in FACS sorting buffer (1 × PBS with 1% bovine serum albumin (BSA (Sigma-Aldrich, Cat# A9418-100G) for subsequent sorting. FlowJo (v10) software was used for analyzing the flow cytometry data.

Renal subcapsular transplantation of mouse MSX1⁺ hindlimb cells

E10.5/E11.5/E13.5/E14.5/E16.5 *Msx1*^{P2A-tdTomato} hindlimbs were dissected and transferred to PBS. After centrifuging and removing the supernatant, 0.25% Trypsin-EDTA was added to digest these limb buds into single cells.

Following incubation at 37 °C for 5 min, digestion was terminated by adding the complete medium. After centrifugation at 450 g for 3 min, the collected cells were resuspended in the FACS sorting buffer. Flow cytometry was performed on BD FACS Aria II, with wt hindlimb cells being used as a negative control. 1.5×10^5 sorted MSX1⁺ cells at all stages were embedded in collagen I (Advanced BioMatrix, Cat# P5005) and incubated at 37 °C for 24 h in the complete medium. Before transplantation, clumps of these MSX1⁺ cells were stripped from the collagen. Then 8-week C57BL6 wt mice were anesthetized by an intraperitoneal injection of Avertin (1.25 g 2, 2, 2-Tribromoethanol (Sigma-Aldrich, Cat# T48402) + 2.5 mL 2-Methyl-2-butanol (Sigma-Aldrich, Cat# 721123) + 97.5 mL UP H₂O) and the skin was disinfected with 75% ethanol. After making a 0.5 cm longitudinal incision in the upper skin and muscle, the kidney was observed and a small incision was made near the kidney pole to separate the capsule from the renal parenchyma (Nakao et al., 2007). Then clumps of MSX1⁺ cells were transplanted into the kidney capsule under a dissecting microscope. Finally, the mice were placed in sterile cages after the skin was closed with Michel's clamps. Three weeks post-transplantation, grafts were dissected and fixed in 4% paraformaldehyde (PFA) at 4 °C for 24 h and then dehydrated in 30% sucrose at 4 °C for more than 48 h. Grafts were then sectioned under a -20 °C condition to get frozen sections and stained by H&E (Solarbio, Cat# G1120-3), Masson (Solarbio,

Cat# G1346), alizarin red (Sigma-Aldrich, Cat# A5533), and Alcian blue (Sigma, Cat# A3157) to demonstrate bone and cartilage differentiation using manufacturer's protocol.

Articular cartilage repair

The method of isolating compact bone-derived MSCs has been described previously in detail (Zhu et al., 2010). Mouse MSX1⁺ cells, MSCs, and the differentiated human cells were embedded in collagen I and incubated at 37 °C in the corresponding medium (mouse MSX1⁺ cells were cultured in the complete medium, MSCs were cultured in α -MEM (Gibco, Cat# C12571500BT) containing 10% FBS and 1% Pen-Strep, human day 8 cells were cultured in LM medium) for 24 h before transplantation. Cell aggregates needed to be dissected with part of collagen I before the defect sites were prepared.

8 weeks old C57BL6 male mice were used in the surgery to make cartilage lesions. The transplantation protocols had been described previously in detail (Fitzgerald, Rich, et al. 2008). Briefly, the mice were anesthetized by an intraperitoneal injection of Avertin, the hair was clipped over the right knee, then the skin was disinfected, and the animals were placed under a dissecting microscope. A small (0.5-1 cm) skin incision was made above the patella, then the joint capsule was opened, and the patella was luxated laterally to expose the trochlear groove articular surface. For

the full-thickness lesion, a circular 0.8 mm defect was conducted in the cartilage with a 23G needle using a circular motion until the subchondral bone was reached (or blood appeared flowing removal of the needle). The cell aggregate was then removed into the cartilage defect. The joint capsule was closed with absorbable 8-0 suture and the skin was closed with a 4-0 suture. The mice were allowed to recover in clean, corncob-lined boxes. There was no evidence of mice lameness or systemic effects for the duration of the experiment. Three or eight weeks later, the transplanted femurs were isolated, and histological analysis was followed.

Immunostaining

Before staining, frozen sections (6 μm) of renal transplantation were washed in PBS gently for 3 min. After being treated with antigen retrieval solution (Solarbio, Cat# C1035), permeabilization was conducted with 0.3% PBST (0.3% Triton X-100 in PBS) (except for membrane proteins), followed by blocking with 5% BSA. Then sections were stained with anti-SOX9 (HUABIO, Cat# ET1611-56, 1: 200), COL II (Invitrogen, Cat# MA5-12789, 1: 200), RUNX2 (HUABIO, Cat# ET1612-47, 1: 200), SP7 (Abcam, Cat# ab209484, 1:200), NESTIN (HUABIO, Cat# R1510-19, 1: 200), S100 β (HUABIO, Cat# ET1610-3, 1: 200), CD31 (Invitrogen, Cat# 11-0311-82, 1: 200), CD34 (HUABIO, Cat# ET606-11, 1: 200), SCA-1 (Invitrogen, Cat# 11-5981-82, 1: 100) antibodies at 4 °C overnight. After

washing in PBS (3×5 min), secondary antibodies were incubated for 1 h at room temperature (except for the direct staining of SCA-1 and CD31 staining). Slides were mounted with DAPI (ZSGB-BIO, Cat# ZLI-9557) when secondary antibodies had been cleaned with PBS (3×5 min).

Paraffin sections ($5 \mu\text{m}$) of articular repair samples were first dewaxed in xylene for 20 min, then rehydrated with 100%, 95%, 80%, 70%, and 50% alcohol gradients (each for 5 min) and immersed in PBS for 3 min. Later, these slides were treated with an antigen retrieval solution of pepsin (Sigma-Aldrich, Cat# R2283) and incubated at 37°C for 30 min. Next, endogenous peroxidase was removed by 3% H_2O_2 for 15 min. When sections had been washed with PBS (3×5 min), slides were stained with anti-COL II antibody at 4°C overnight. A secondary antibody (ZSGB-BIO, Cat# ZLI-9018) was added to the slides and incubated at room temperature for 1 h when the primary antibody had been removed by PBS (3×5 min). After cleaning the secondary antibody, DAB and hematoxylin staining were followed. At last, the sections were dehydrated with 80%, 95%, 100% alcohol, and xylene successively, then sealed with Mounting Medium. Alcian blue staining was also required on the rehydrated slides. Images were acquired with Leica TCS SP5II, Olympus VS200, and Wisleap WS-10 and analyzed by Image J software.

Single-cell preparation and scRNA sequencing of E10.5 MSX1^+ cells

About 20 hindlimb buds were dissected from E10.5 *Msx1*^{P2A-tdTomato} mice. These limb buds were dissociated into single cells first and resuspended in PBS with 1% BSA. The sorted MSX1⁺ cells were both counted and adjusted to the concentration of about 1×10^6 /mL. Then the suspension was centrifuged at 550 g for 5 min at 4 °C and repeated twice. Cells were counted and cell viability was confirmed by Countess II Automated Cell Counter (Thermo Fisher, Cat# AMQAX1000). Samples were then used for single-cell RNA sequencing (scRNA-seq) with the 10x Genomics system (Library preparation and sequencing were performed by Berry Genomics Inc, Beijing).

Processing of scRNA-seq raw sequencing data

The CellRanger software was obtained from the 10x Genomics website (<https://www.10xgenomics.com/software>). Alignment, filtering, barcode counting, and UMI counting were performed with the cell ranger count module to generate a feature-barcode matrix. scRNA-seq data of E10.5 MSX1⁺ mesenchymal progenitors has been submitted to the GEO database (GEO: GSE232586).

Reduction, clustering, and identification of differentially expressed genes in E10.5 mouse limb

The feature-barcode matrix was subsequently processed using R and

Seurat v4.2 package. We discarded cells that have unique features of fewer than 2200 and have counts of fewer than 6000. Subsequently, low-quality genes were identified as being expressed in less than 3 cells. The gene expression levels for each cell were normalized by the total expression, multiplied by a default size factor of 10,000, and log-transformed. Cell cycle effects were regressed with Seurat's function "ScaleData" using cell cycle markers. The dimensionality of the data was reduced by principal component analysis (PCA) (17 components) first and then data were clustered and visualized with UMAP (Uniform Manifold Approximation and Projection) on the 17 principal components (resolution = 0.7). Finally, the 7 cell clusters (LM, LPM, AER, SMCs, MCs, VECs, and Others) were then identified through cell-cluster-specific gene markers.

LPM and LM pseudotime analysis of mouse and human limb bud

Pseudotemporal ordering of LPM and LM cells was done with Monocle 3 v1.2.9. The data was further processed using UMAP with default parameters. A cluster graph was then created and partitioned to deduce disconnected trajectories. Subsequently, a principal graph in the low-dimensional space was generated and the pseudotime was calculated as the geodesic distance.

ScRNA-seq analysis of human 5 WPC limb

The processing of scRNA-seq raw sequencing data was consistent with mouse E10.5 data. The feature-barcode matrix was subsequently processed using R and Seurat v4.2 package.

We discarded cells that had unique features of fewer than 1,000 and had mitochondrial count percentages of more than 10%, low-quality genes were identified as being expressed in less than 3 cells. The gene expression levels for each cell were normalized by the total expression, multiplied by a default size factor of 10,000, and log-transformed. Cell cycle effects were regressed with Seurat's function "ScaleData" using cell cycle markers. Human 5 WPC limb scRNA-seq data of cs13 and cs15 (He et al., 2021) were integrated into a whole dataset according to Seurat's function "RunCCA". The dimensionality of the data was reduced by principal component analysis (PCA) (30 components) first and then data were clustered and visualized with UMAP on the 30 principal components (resolution = 0.4). Then we identified hindlimbs and separated LPM, and LM for downstream pseudotime analysis. AER cells were also characterized for Cellchat analysis.

Transcriptome analysis by RNA sequencing

RNA sequencing was conducted to investigate the gene expression profiles of the differentiated human day 8 $MSX1^+PDGFRA^{low}$ and $MSX1^+PDGFRA^+$ cells for FGF8+10 and Mock groups (n = 2), and 150

base pair paired-end reads were generated. The adaptors and low-quality reads from the raw reads of each sample were trimmed to obtain clean reads. The clean reads were mapped against the human genome (GRCh38) using HISAT2 v2.1.0. The expression level of each gene was quantified guided by reference annotation (GRCh38.104) using feature Counts v1.6.4. The PCA was analyzed and visualized by DESeq2 packages. RNA-seq data have been submitted to the GEO database (GEO: GSE232586).

Cell culture of hPSCs

Before the thawing, culture, and passaging of hPSCs, cell culture plates were coated with Matrigel (Corning, Cat# 354230) with a concentration of 1 mg/12mL in DMEM/F12 (Gibco, Cat# C11330500) at 37 °C for at least 1 h. Then the H1-hPSCs with clumps state were cultured and maintained in Pluripotency Growth Master 1 medium (PGM1, CELLAPY, Cat# CA1007500) in an incubator at 37 °C with 5% CO₂. When attaining subconfluency, the cells were dissociated into small clumps with TrypLE (Gibco, Cat# 12605028) and suspended in a PGM1 medium containing 10 μM Y27632 (MCE, Cat# HY10583). Y27632 was added to the PGM1 medium on the day of cell thawing and passage, and the fresh medium without Y27632 was replaced the next day. The medium was changed every day.

Generation of human *MSX1*^{P2A-tdTomato} reporter cell line

The P2A-tdTomato-Loxp-PGK-Puro-Loxp cassette was inserted into the site before the stop codon of human *MSX1* by homology-directed repair (HDR) to achieve bicistronic expression of *MSX1* and tdTomato. The *MSX1* 5' arm (642 bp) and 3' arm (503 bp) were amplified using PCR. P2A-tdTomato-Loxp-PGK-Puro-Loxp cassette, synthesized by BGI Genomics Co., Ltd, together with 5' arm and 3' arm were cloned to PUC19 plasmid to form the donor plasmid. The designed guide RNAs (gRNA, 5'-GCATGTACCACCTGACATAG-3') were combined with the *MSX1* stop codon and its 17 bp sequence of the 5' arm. These gRNA oligos were annealed into double-stranded and cloned into PX330 vector expressing Cas9 cassette, and then PX330-*MSX1*gRNA-Cas9 plasmid was constructed. 1 µg linearized donor plasmid with 1 µg PX330-*MSX1*gRNA-Cas9 plasmid were co-transfected into 1×10^5 H1-hPSCs by Lipofectamine stem reagent (ThermoFisher, Cat# STEM00015) using manufacturer's protocol. Puromycin screening was performed two days after transfection, and the screening lasted until no cell death was observed. When the confluency reached 80%, puromycin-resistant cells were cultured in 96-well plates to isolate the monoclonal cells and then expanded in 24-well plates or 12-well plates. To identify the genotypes of these monoclonal cell populations, the genome of screened cells was extracted using a Genomic Extraction kit (TIANGEN, Cat# DP304-03). The genome was

subsequently identified by PCR and sequencing. Three monoclonal cell lines were administered genotypic identification and one of them was stained by anti-OCT4 (Abcam, Cat# ab19857) and SOX2 (Abcam, Cat# ab97959) antibodies. It was proved that these cells maintained pluripotency during the process of cell line construction. Primers used in this section were listed in Table S2.

Differentiation of LPM and LM-like cells

The *MSX1^{P2A-tdTomato}*-hPSCs were digested by Accutase (STEM CELL, Cat# 07920) for 4 min into single cells. After counting, cells were suspended with PGM1 medium containing Y27632 and seeded in a new Matrigel-coated culture dish with a density of 1×10^4 cells per square centimeter. The medium containing Y27632 was removed and replaced with PGM1 medium the next day. The cells were washed with a wash medium (DMEM/F12 containing 3 mg/mL BSA and 1% Pen-Strep) to remove the residual PGM1 medium. Then cells were washed with CDMi basic medium (50% F12 (Gibco, Cat# 31765-035) + 50% IMDM (Gibco, Cat# 31980-030) + 1 mg/mL Poly(vinyl alcohol) (Sigma, Cat# P8136) + 450 μ M Monothioglycerol (Sigma, Cat# M6145) + 0.7 μ g/mL Insulin (Solarbio, Cat# I8830) + 15 μ g/mL transferrin (Sigma, Cat# T0665) + 1% Pen-Strep + 1% Lipids concentrate (Gibco, Cat# 11905-031)). The MPS medium (CDMi medium + 50 ng/mL Activin A (Solarbio, Cat# P00101) +

6 μ M CHIR99021 (MCE, Cat# HY-10182A) + 40 ng/mL BMP4 (R&D systems, Cat# 314-BP/CF) + 10 ng/mL FGF2 (Peprotech, Cat# 100-18B)) was added at day 0. On day 1, the MPS medium was replaced by LPM medium (CDMi medium + 60 ng/mL BMP4 + 1 μ M CHIR99021). This process lasted for 3 days. The day 4 LPM was in an immature state and needed to continue differentiation in the LM medium (CDMi medium + 500 ng/mL FGF8 (MCE, Cat# HY-P70533) + 500 ng/mL FGF10 (MCE, Cat# HY-P4088)). When the day 4 cells had been digested with TryPLE for 2 min, the wash medium was added to terminate the digestion. After centrifugation at 450 g for 3 min, the cells were resuspended in the LM medium and inoculated into Poly (2-hydroxyethyl methacrylate) (Sigma, Cat# P3932)-treated low-adhesion U-bottom 96-well plates with 2.5×10^5 cells per well.

To investigate the efficiency of different growth factors in promoting cell maturation to LM, CHIR99021 (6 μ M) and FGF2 (500 ng/mL) were also added to the CDMi medium before inoculation, as CDMi medium was set as Mock control. Cells were centrifuged 300 g for 6 min and incubated at 37 °C for 24 h to promote pellet formation. On day 5 of collection, the pellets were placed on a low-adhesion 60 mm plate treated with Poly(2-hydroxyethyl methacrylate) and cultured for 3 days using the LM medium.

qRT-PCR

Total RNA was extracted from cells using Trizol reagent (ThermoFisher, Cat# 15596026) according to the manufacturer's instructions. cDNAs were prepared using the PrimeScript RT reagent kit (Takara Biomedical Technology, Cat# RR047A). The cDNAs were then used as templates for qPCR analysis with gene-specific primers. The qPCR was performed using a CFX384 real-time PCR system (BIO-RAD, USA). The cycle parameters were as follows: denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s. The expression level of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method. Primers used in qRT-PCR were listed in Table S3.

Osteogenic and chondrogenic differentiation assays

5×10^4 cells of the E10.5 subsets ($MSX1^+PDGFRA^{high}$, $Msx1^+PDGFRA^{medium}$, and $MSX1^+PDGFRA^{low}$ cells) or the isolated MSCs cells were added into a 24-well plate to perform the osteogenic differentiation, the corresponding differentiation protocol has been described previously (Zhu et al., 2010). For the sorted human cells, 2.5×10^5 digested cells were cultured in a hole of 24-well plates. The osteogenesis process was performed in the osteogenic medium (α -MEM + 10% FBS + 100 nM dexamethasone (MCE, Cat# HY-14648) + 50 μ g/mL L-ascorbate acid 2-phosphate (Sigma, Cat# A8960) + 10 mM β -glycerophosphate (Millipore, Cat# 35675-50GM) + 1% Pen-Strep) for

three weeks (medium was changed every 3 days) and identified by alizarin red staining.

The chondrogenic differentiation of 1×10^5 isolated primary MSX1⁺ cells and MSCs was performed by micromass methods (ten Berge et al., 2008) and lasted for 3 weeks (the medium was changed every 3 days). The chondrogenic medium contained high glucose DMEM (Gibco, Cat# C11995500BT) supplemented with 10 ng/mL TGFβ3 (Peprotech, Cat# 100-36E), 30 ng/mL BMP4, 100nM dexamethasone, 50 μg/mL L-ascorbic acid-2-phosphate, 1 mM sodium pyruvate (Gibco, Cat# P5280), 40 μg/mL L-proline (Sigma-Aldrich, Cat# P0380) and 1 × ITS cell culture supplement (Thermofisher, Cat# 41400045). For the chondrogenesis of differentiated human cells, 1×10^5 cells were added into a sterile well of PCR 8-tube with culture medium (CDMi medium + 10 nM dexamethasone + 30 ng/mL BMP4 + 50 μg/mL L-ascorbate acid + 10 ng/mL TGFβ3 + 1 mM sodium pyruvate + 40 μg/mL proline + 1 × ITS). Four weeks later, these chondrogenic spheres were fixed in 4% PFA at 4 °C for 24 h and then dehydrated in 30% sucrose at 4 °C overnight. Alcian blue staining would be conducted to identify the chondrocytes.

Supplemental References

Fitzgerald, J., Rich, C., Burkhardt, D., Allen, J., Herzka, A.S., and Little,

C.B. (2008). Evidence for articular cartilage regeneration in MRL/MpJ mice. *Osteoarthritis Cartilage* 16, 1319-1326.

10.1016/j.joca.2008.03.014.

He, J., Yan, J., Wang, J., Zhao, L., Xin, Q., Zeng, Y., Sun, Y., Zhang, H., Bai, Z., Li, Z., et al. (2021). Dissecting human embryonic skeletal stem cell ontogeny by single-cell transcriptomic and functional analyses. *Cell Res* 31, 742-757. 10.1038/s41422-021-00467-z.

Nakao, K., Morita, R., Saji, Y., Ishida, K., Tomita, Y., Ogawa, M., Saitoh, M., Tomooka, Y., and Tsuji, T. (2007). The development of a bioengineered organ germ method. *Nat Methods* 4, 227-230. 10.1038/nmeth1012.

ten Berge, D., Brugmann, S.A., Helms, J.A., and Nusse, R. (2008). Wnt and FGF signals interact to coordinate growth with cell fate specification during limb development. *Development* 135, 3247-3257. 10.1242/dev.023176.

Zhu, H., Guo, Z.K., Jiang, X.X., Li, H., Wang, X.Y., Yao, H.Y., Zhang, Y., and Mao, N. (2010). A protocol for isolation and culture of mesenchymal stem cells from mouse compact bone. *Nat Protoc* 5, 550-560. 10.1038/nprot.2009.238