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Supplemental information

Time- and cell-specific activation of BMP signaling restrains chondrocyte hypertrophy

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Figure S1, Related to Figures 1 and 2

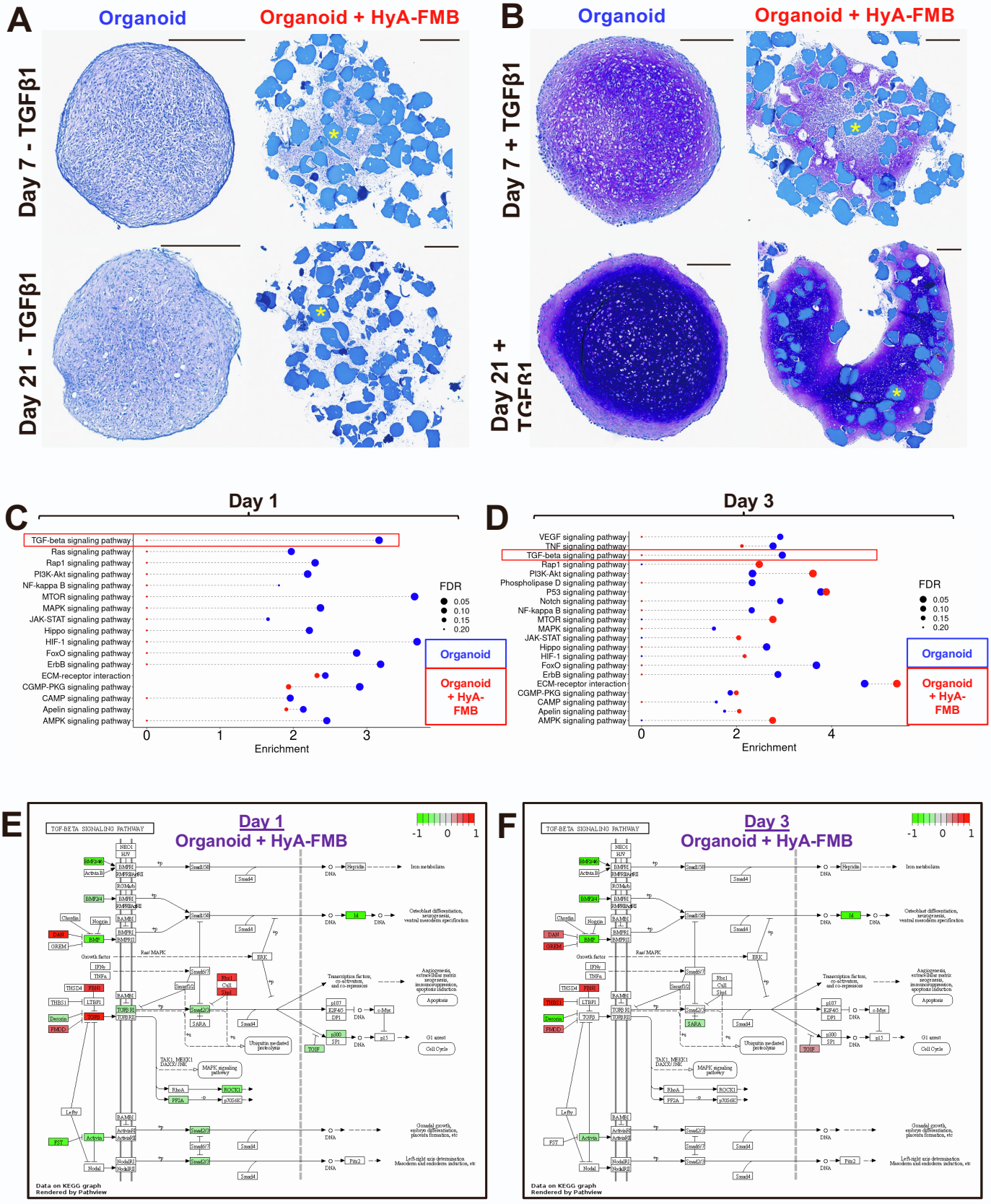


Figure S2, Related to Figures 2 and 3

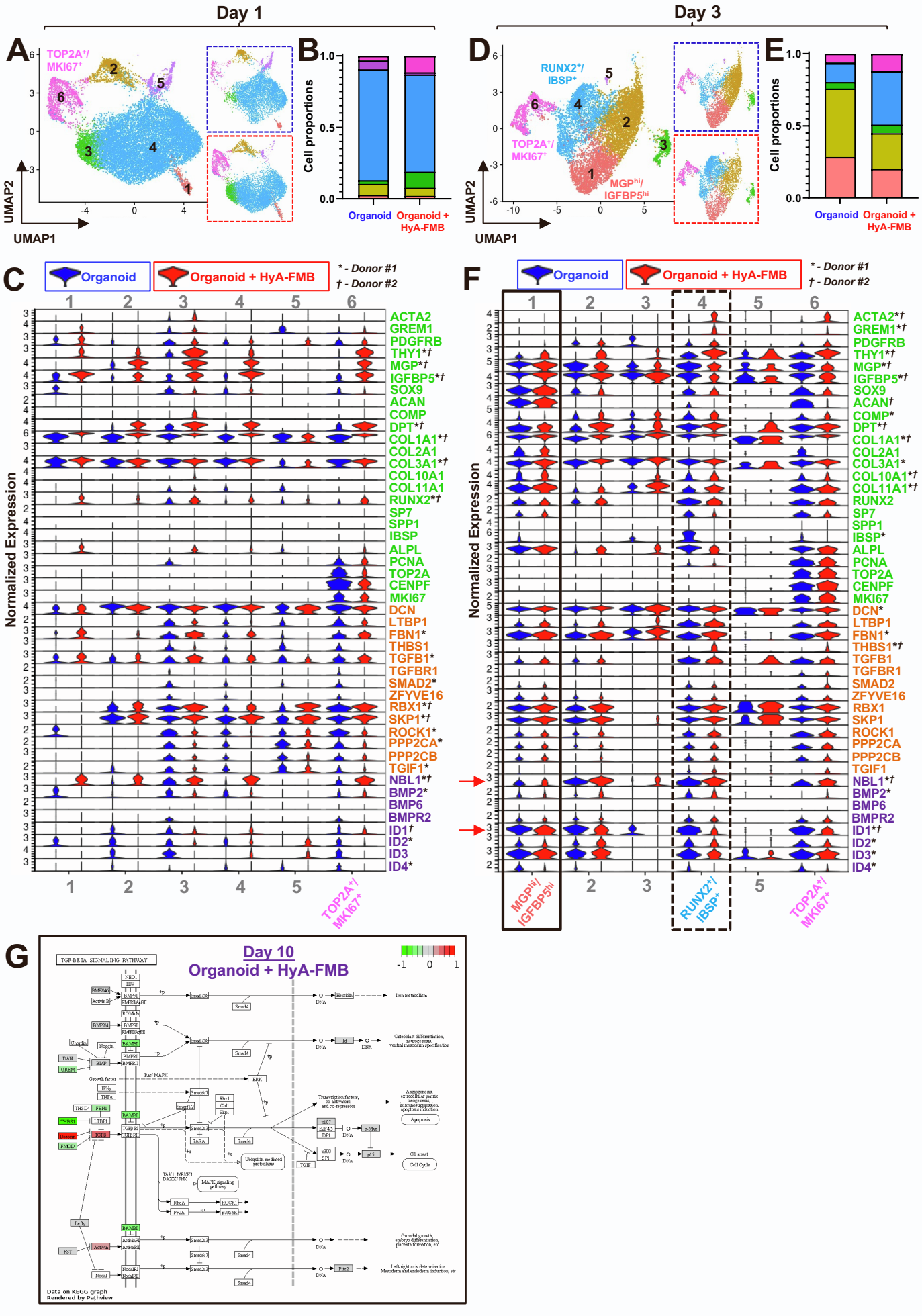


Figure S3, Related to Figure 4

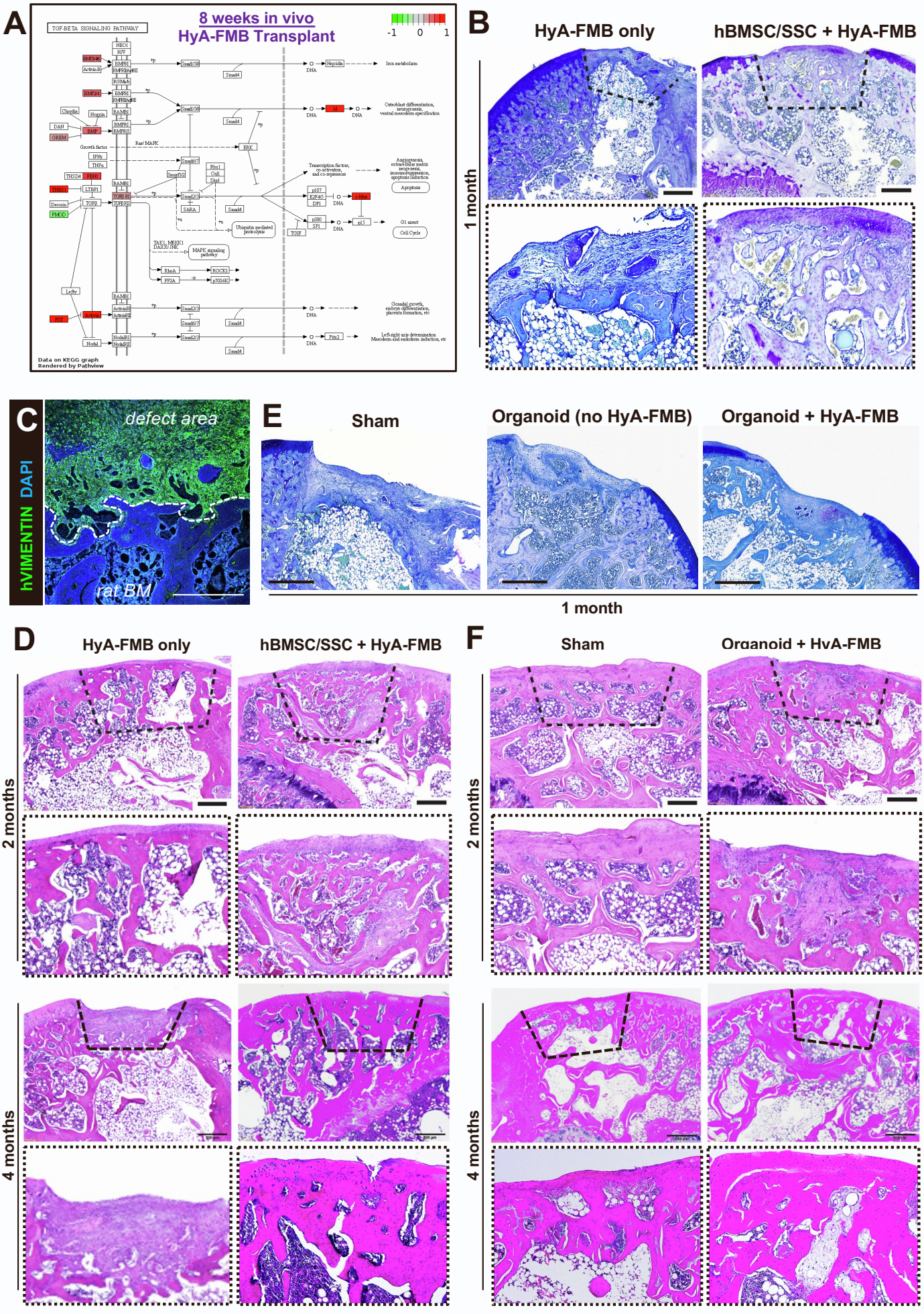


Figure S4, Related to Figure 5

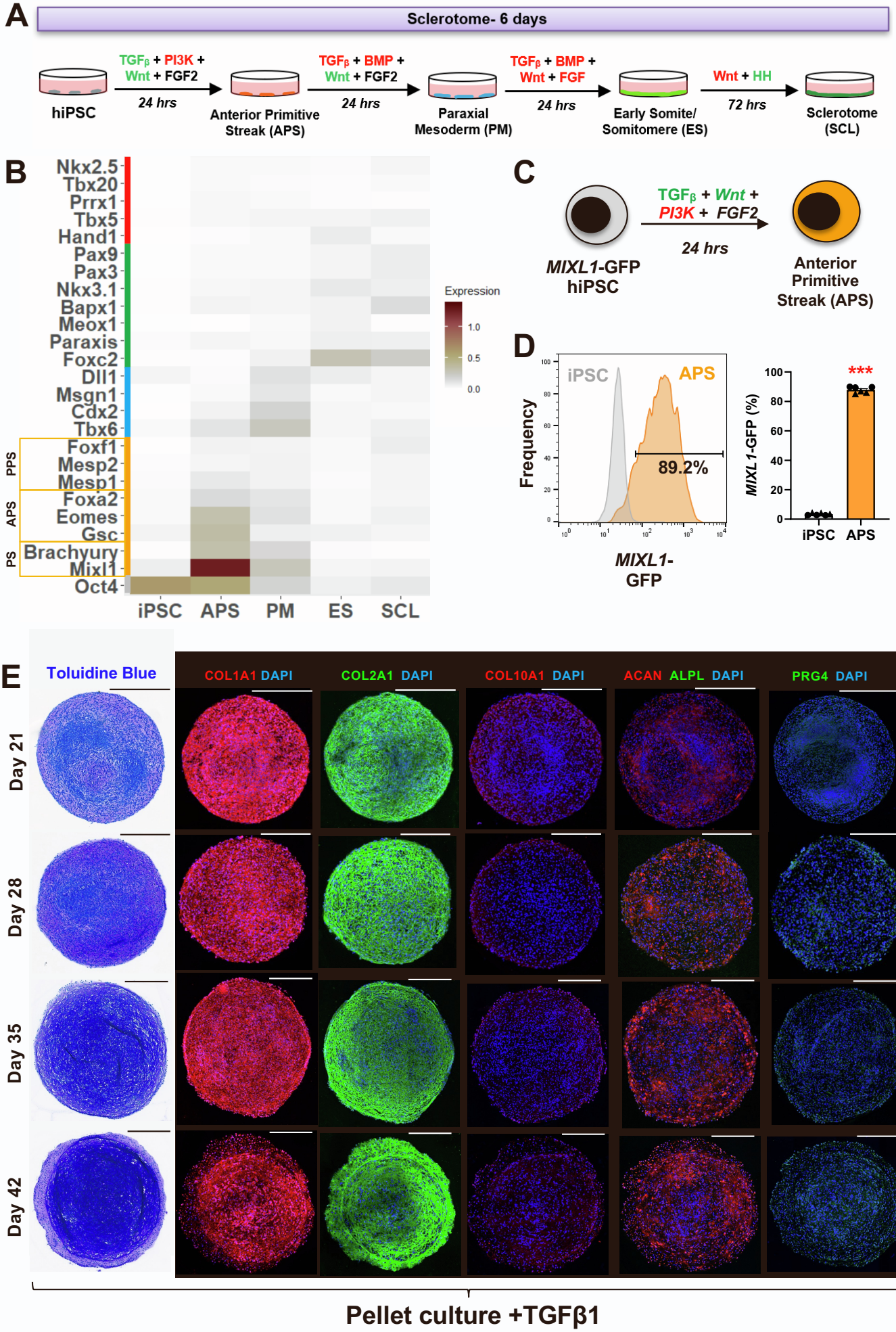
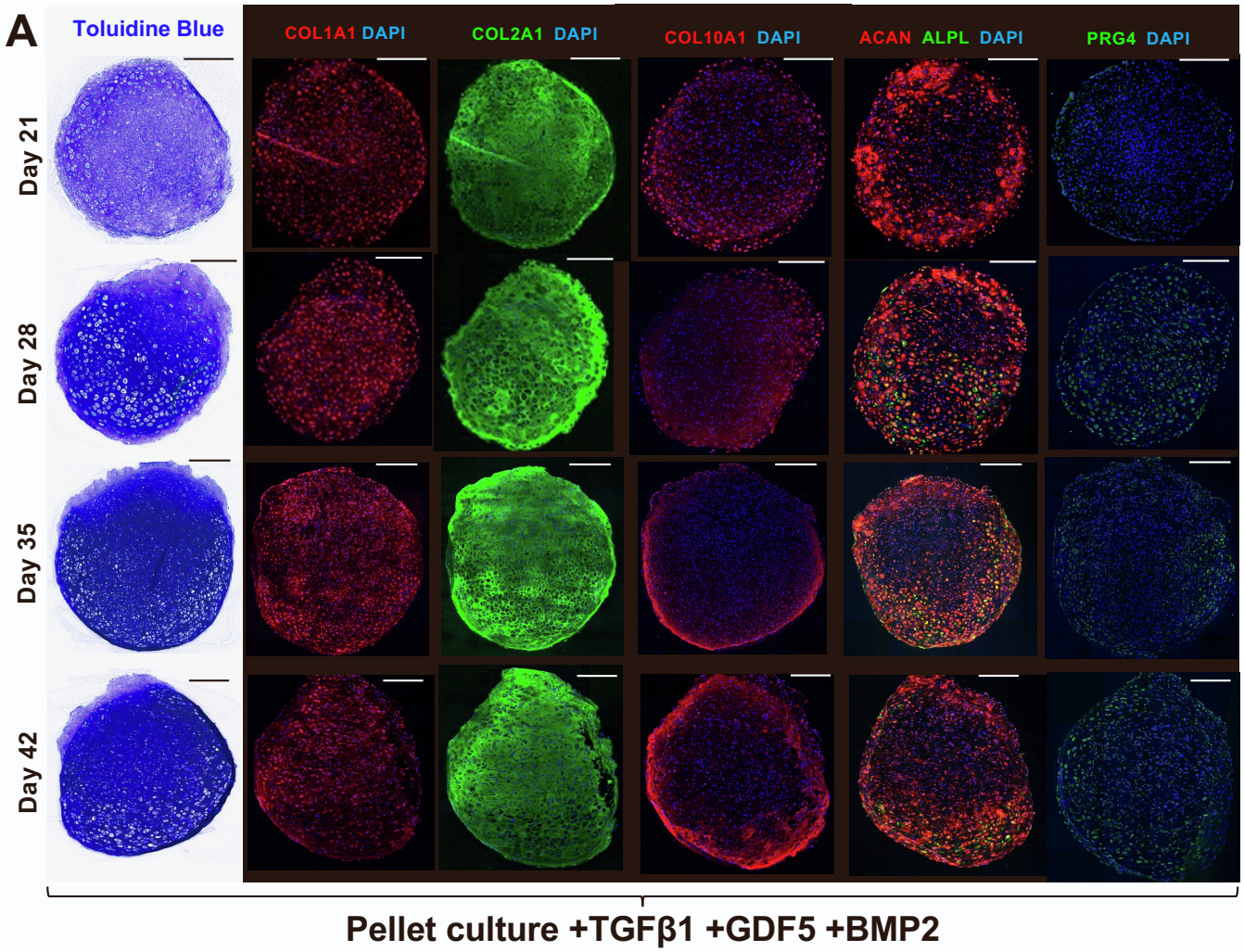
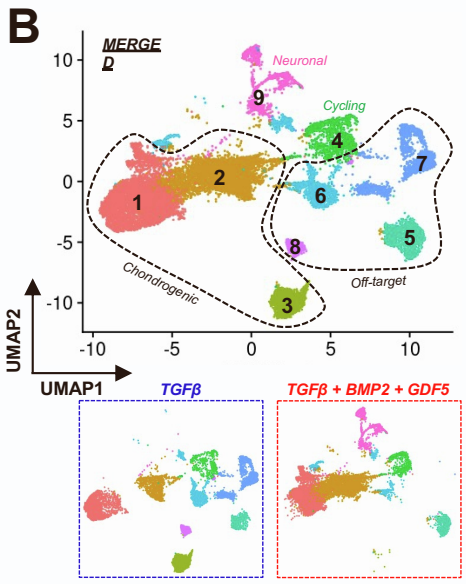
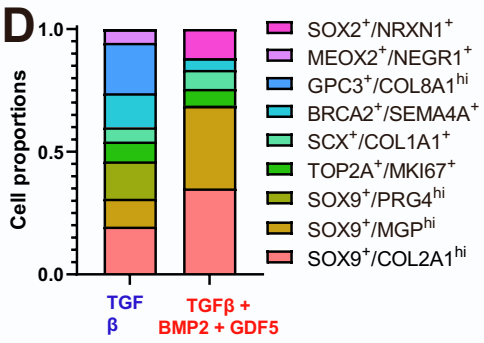


Figure S5, Related to Figure 5







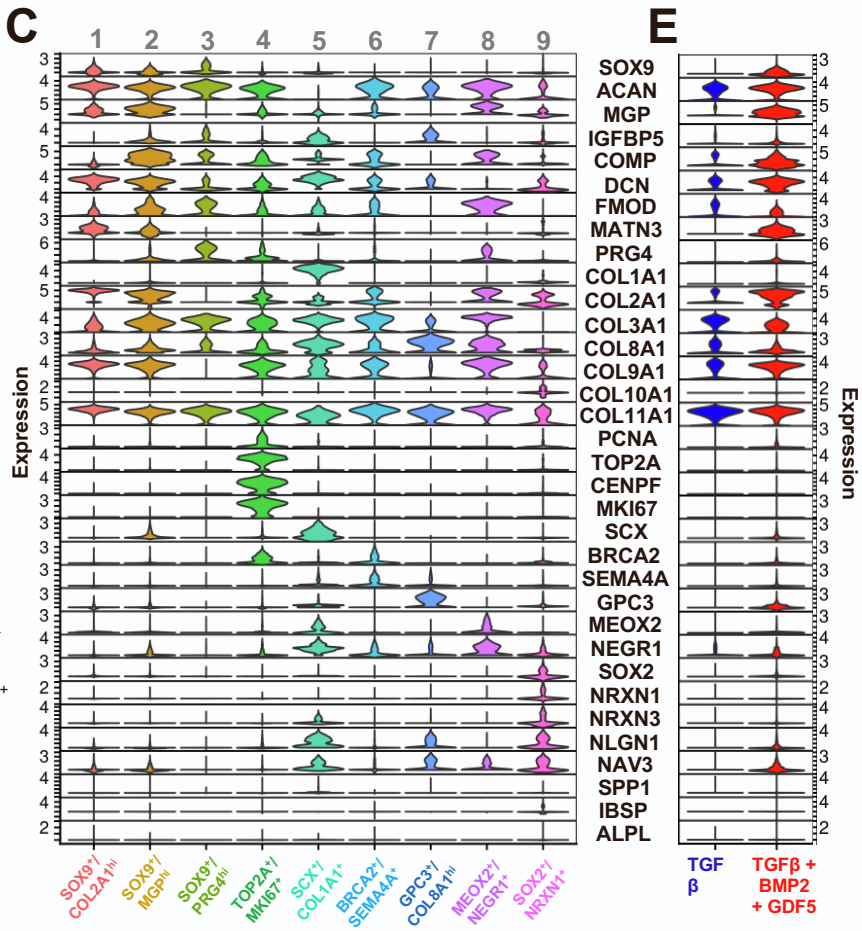


Figure S6, Related to Figure 6

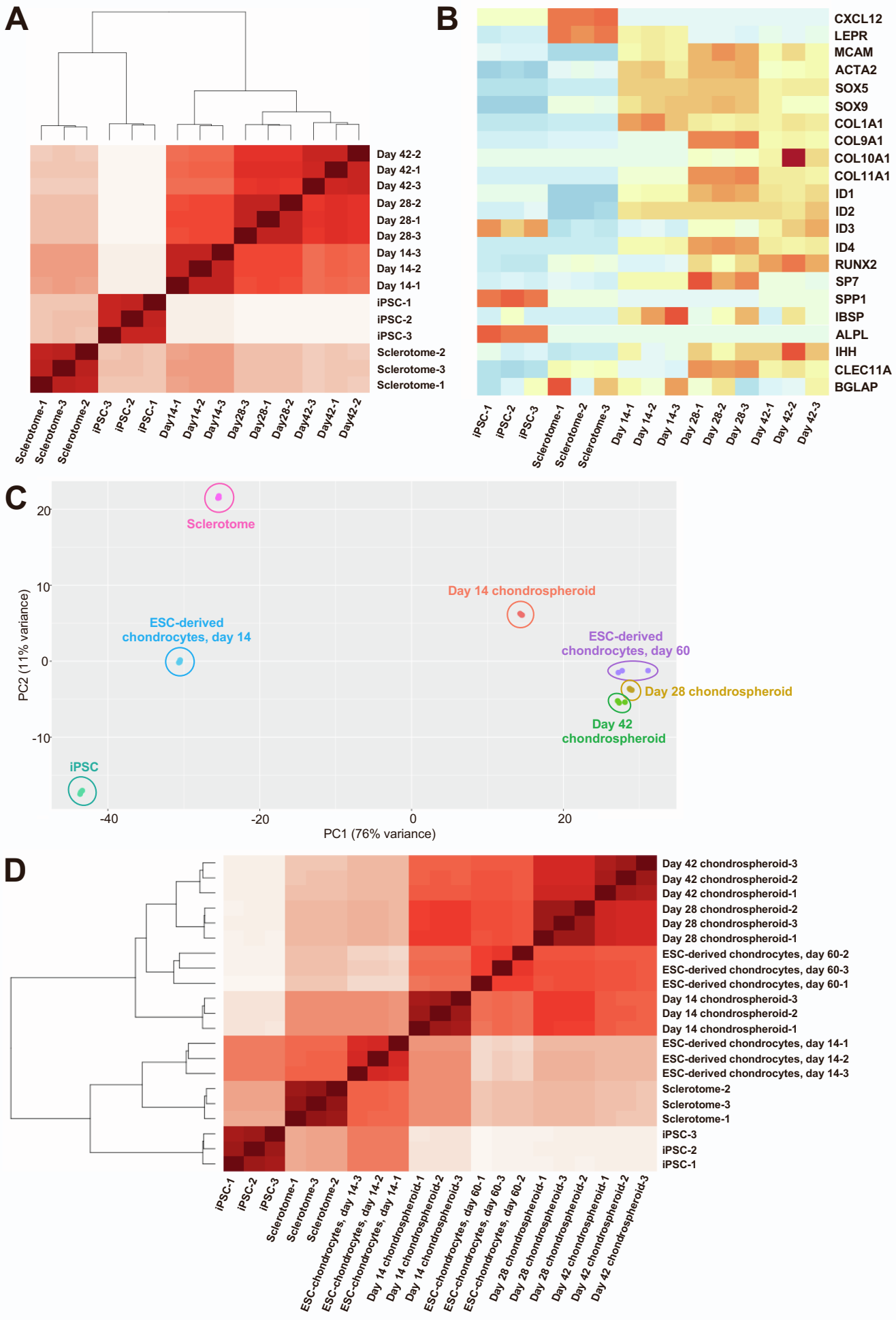
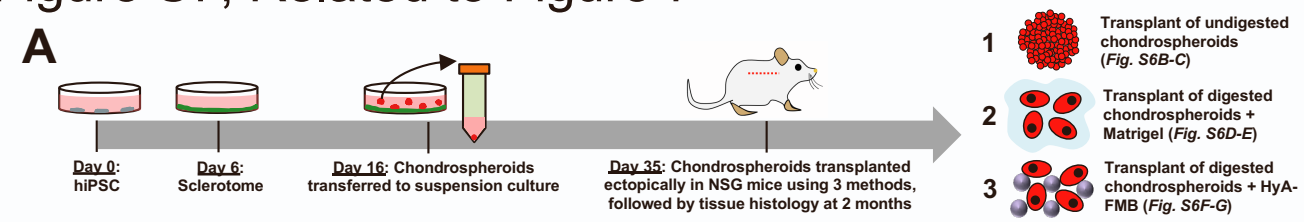
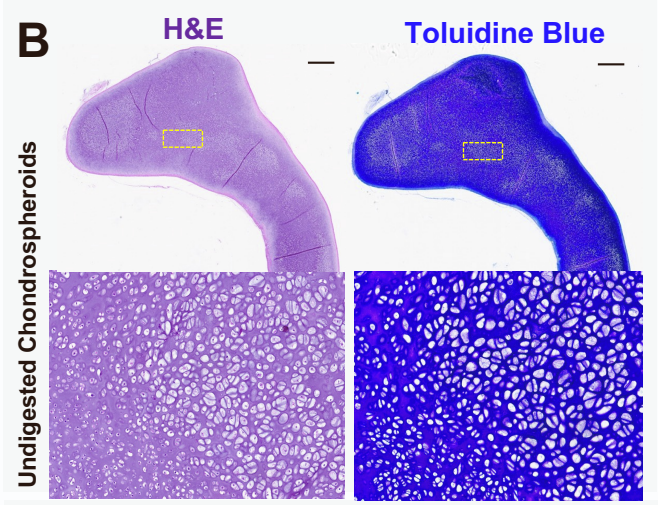
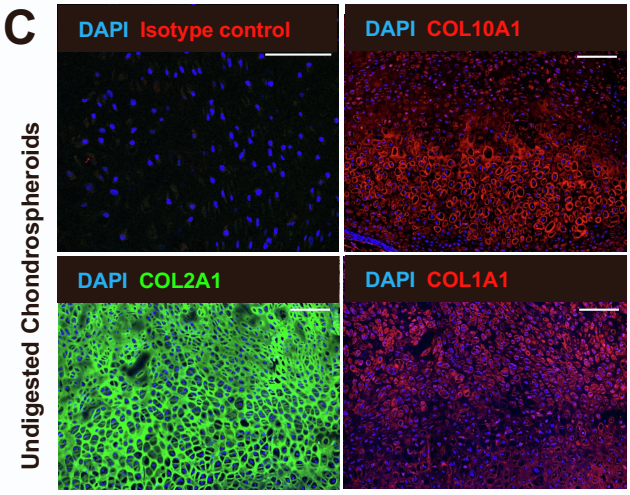
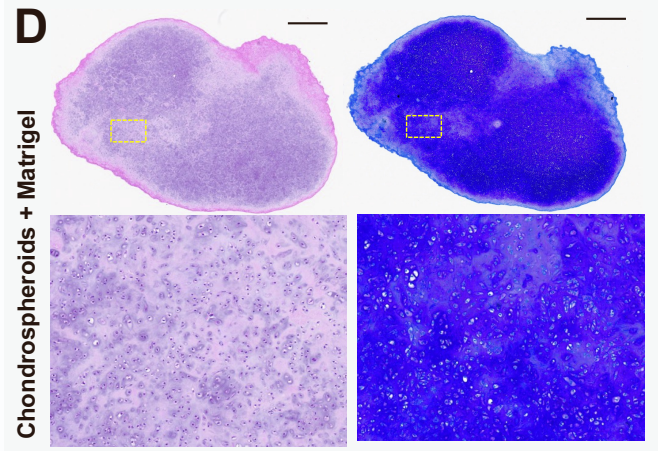


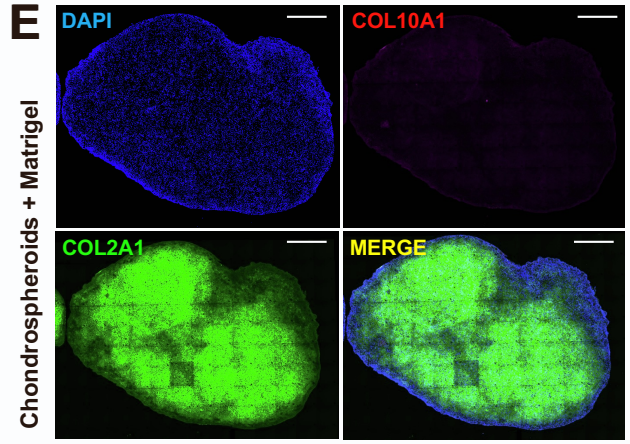
Figure S7, Related to Figure 7

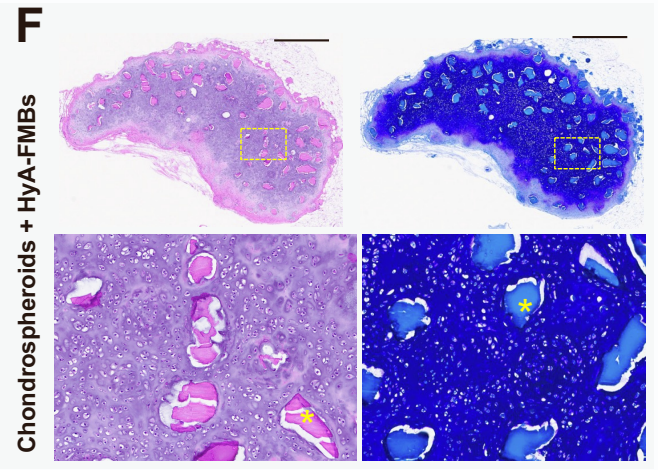


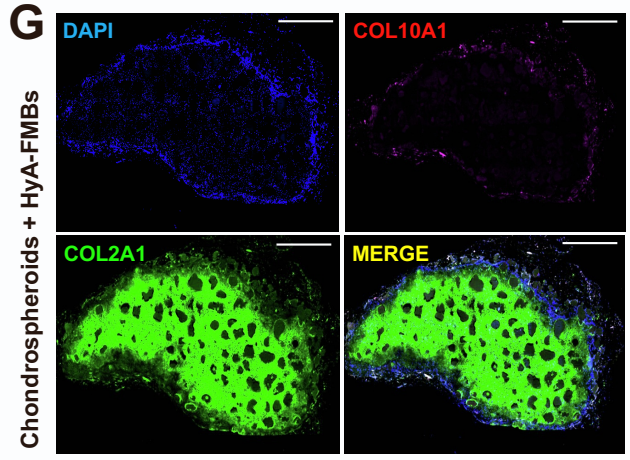












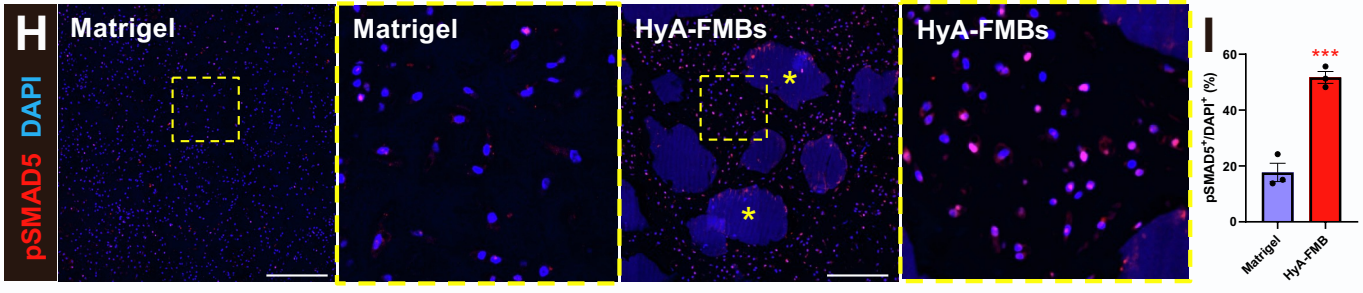
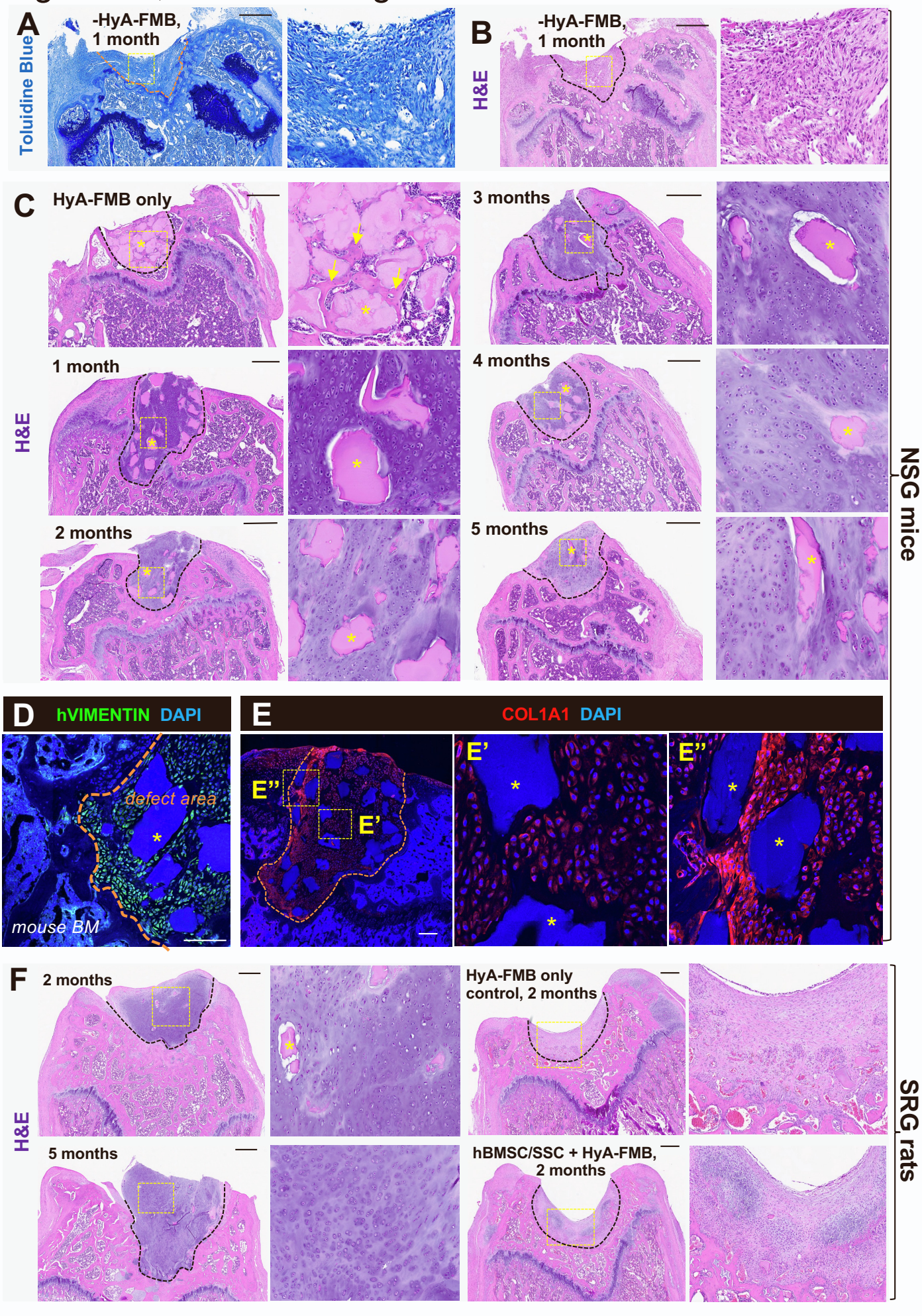


Figure S8, Related to Figure 7



Supplementary Figure Legends

Figure S1, Related to Figures 1 and 2. HyA-FMBs promote early expression of extracellular matrix genes.

(A-B) hBMSCs/SSCs were used to make cell pellet cultures without HyA-FMBs (A, left panel; B: left panel) and with HyA-FMBs (A, right panel; B right panel). Pellets were differentiated in medium without TGFβ1 (A) and with TGFβ1 (B) for 7 (A and B top panels) and 21 days (A and B bottom panels). No metachromatic staining (purple) indicative of cartilage was noted at either 7 or 21 days cultures (A). Metachromatic staining was initiated by 7 days, and very intense at 21 days in both types of cultures due to the high concentration of glycosaminoglycans (B). Scale bars, 300μm. Asterisks (*) indicate HyA-FMBs.

(C-D) Gene enrichment analysis of KEGG signal transduction pathways from differentially expressed genes among control and HyA-FMB organoids (logfc>0.25) at day 1 (C) and day 3 (D) of chondrogenic differentiation.

(E-F) Gene enrichment analysis of KEGG TGFβ signaling pathway from differentially expressed genes (logfc>0.25) in HyA-FMB organoids compared with controls at day 1 (E) and day 3 (F) of chondrogenic differentiation.

(G-H) Immunofluorescence of COL1A1 (pink) and COL2A1 (green) in control organoids (G) and HyA-FMB organoids (H) from day 3 of chondrogenic differentiation. Scale bars, 100μm (G) and 300μm (H).

Figure S2, Related to Figures 2 and 3. HyA-FMBs suppress BMP signaling early in chondrogenic differentiation, but restore BMP signaling in MGP/IGFBP5-enriched chondrogenic cells.

(A-F) Cluster analyses from scRNA-seq of control (blue) and HyA-FMB (red) organoids at day 1 (A-C) and day 3 (D-F) of chondrogenic differentiation. Control and HyA-FMB datasets at each timepoint were normalized and integrated using Seurat. Data is shown from donor #1.

(A, D) UMAP representation of integrated control and HyA-FMB datasets with annotated clusters. Split UMAPs are shown on the right for control organoids (blue dashed outline) and HyA-FMB organoids (red dashed outline).

(B, E) Bar chart depicting proportion of cell clusters in control and HyA-FMB datasets.

(C, F) Violin plots depicting gene expression split by experimental condition (blue violin plots are control organoids, red violin plots are HyA-FMB organoids) from integrated datasets at day 1 (C) and day 3 (F) of chondrogenic differentiation. Genes associated with TGFβ signaling (orange), BMP signaling (purple), and chondro-osteogenesis (green) are shown. Arrows depict notable gene expression differences (logfc>0.25, $p<0.05$) between $MGP^{hi}/IGFBP5^{hi}$ cluster (solid black box)

and RUNX2^{+}/IBSP^{+} cluster (dashed black box). Global significance (logfc>0.25, p<0.05) between control and HyA-FMB organoids is shown with an asterisk (*) for donor #1 and dagger (†) for donor #2.

(G) Gene enrichment analysis of KEGG TGFβ signaling pathway from differentially expressed genes within MGP^{hi}/IGFBP5^{hi} cluster (logfc>0.10) in HyA-FMB organoids compared with controls at day 10 of chondrogenic differentiation.

Figure S3, Related to Figure 4. Rat chondral transplantation of hBMSC/SSC/HyA-FMB constructs yields suboptimal chondrogenesis.

(A) Gene enrichment analysis of KEGG TGFβ signaling pathway from differentially expressed genes (logfc>0.25) in transplanted HyA-FMB organoids compared with cultured HyA-FMB organoids from day 10 of chondrogenic differentiation.

(B) Toluidine Blue staining of defect areas (dashed lines) at 1 month-post transplant in SRG rats from HyA-FMB chondral transplants shown in Figure 4F. Scale bars, 500μm. High magnification images shown below each image.

(C) Immunofluorescence analysis of human VIMENTIN to confirm human origin of transplanted hBMSCs/SSCs attached to HyA-FMBs at 2 months-post-transplant. Dashed line indicates boundary separating transplanted cells from rat bone marrow (BM). Nuclei counterstained with DAPI. Scale bar, 500μm.

(D) H&E staining of defect areas (dashed lines) at 2 and 4 months-post transplant in SRG rats from chondral transplants shown in Figure 4F. Scale bars, 500μm. High magnification images shown below each image.

(E) Toluidine Blue staining of defect areas at 1 month-post transplant in SRG rats from sham, control organoid (no HyA-FMBs) and HyA-FMB organoid transplants shown in Figure 4H.

(F) H&E staining of defect areas (dashed lines) at 2 and 4 months-post transplant in SRG rats from organoid chondral transplants shown in Figure 4H. Scale bars, 500μm. High magnification images shown below each image.

Figure S4, Related to Figure 5. A serum-free chondrogenic differentiation strategy beginning with sclerotome induction.

(A) Schematic of sclerotome differentiation strategy from hiPSCs: pathway activators (green), inhibitors (red), and recombinant growth factors (black) were added across 6 days of adherent culture.

(B) Heatmap depicting mRNA expression of hiPSC differentiation to anterior primitive streak (APS), paraxial mesoderm (PM), early somite/somitomere (ES), and sclerotome (SCL) from

qRT-PCR experiments. Orange line indicates primitive streak markers, blue line indicates paraxial mesoderm markers, green line indicates somite/sclerotome markers, and red line indicates lateral and cardiac mesoderm markers. N=3 technical replicates.

(C) Schematic of anterior primitive streak differentiation strategy of MIXL1-GFP hiPSCs: pathway activators (green) and inhibitors (red) and recombinant growth factors (black) were added across 24 hours of adherent culture, followed by FACS analysis.

(D) Representative FACS plot (left) and quantification (right) of MIXL1-GFP expression in MIXL1-GFP hiPSCs differentiated to anterior primitive streak for 24 hours. Each shape represents a biological replicate. Data are mean ± SEM; ***$p < 0.001$, unpaired two-tailed t test.

(E) hiPSC-derived sclerotome cells were pelleted in chondrogenic medium supplemented with TGFβ, followed by Toluidine Blue staining and immunofluorescence analyses of tissues at days 21, 28, 35, and 42. Nuclei counterstained with DAPI. Scale bars, 300μm. Area quantifications are shown in Figure 5A.

Figure S5, Related to Figure 5. A serum-free chondrogenic differentiation strategy beginning with sclerotome induction.

(A) hiPSC-derived sclerotome cells were pelleted in chondrogenic medium supplemented with TGFβ, BMP2, and GDF5, followed by Toluidine Blue staining and immunofluorescence analyses of tissues at days 21, 28, 35, and 42. Nuclei counterstained with DAPI. Scale bars, 300μm. Area quantifications are shown in Figure 5A.

(B-E) hiPSC-derived sclerotome was cultured in chondrogenic medium with TGFβ in combination with BMP2 and GDF5 (chondrospheroid protocol) for 42 days, after which cultures were digested for scRNA-seq.

(B) UMAP representation of integrated datasets from day 42 cultures with annotated clusters. Split UMAPs are shown below for TGFβ- (blue dashed outline) and TGFβ, BMP2, and GDF5- (red dashed outline) treated cultures.

(C) Violin plots of integrated datasets showing marker genes for each cluster.

(D) Bar chart depicting proportion of cell clusters from each dataset, which includes all cell populations (clusters 1-9).

(E) Violin plots depicting global gene expression.

Figure S6, Related to Figure 6. Chondrospheroid transcriptomes reveal a fetal-like chondrogenic identity.

(A) Hierarchical clustering analysis of hiPSC, sclerotome, and chondrospheroid datasets.

(B) Heatmap depicting genes of interest from hiPSC, sclerotome, and chondrospheroid datasets.

(C) PCA of hiPSC, sclerotome, and chondrospheroid datasets, which were batch-corrected and normalized using ComBat-seq to ESC-derived chondrocytes at days 14 and 60 from a previous study[42].

(D) Hierarchical clustering analysis of hiPSC, sclerotome, chondrospheroid, and ESC-derived chondrocyte[42] datasets.

Figure S7, Related to Figure 7. HyA-FMBs promote stable chondrogenesis in subcutaneous chondrospheroid transplants.

(A) Schematic of hiPSC differentiation to sclerotome and chondrospheroids, which were transplanted at day 35 using three methods.

(B) H&E (left) and Toluidine Blue (right) staining of subcutaneous transplants of undigested day 35 chondrospheroids at 2 months-post-transplant in NSG mice. High magnification insets shown below their corresponding images. Scale bars, 500μm.

(C) Immunofluorescence analyses of COL10A1, COL2A1, and COL1A1 from undigested day 35 chondrospheroids at 2 months-post-transplant in NSG mice. Scale bars, 100μm.

(D) H&E (left) and Toluidine Blue (right) staining of subcutaneous transplants of digested day 35 chondrospheroid cells embedded in Matrigel™ at 2 months-post-transplant in NSG mice. High magnification insets shown below their corresponding images. Scale bars, 500μm.

(E) Immunofluorescence analyses of COL10A1 and COL2A1 from digested day 35 chondrospheroid cells embedded in Matrigel™ at 2 months-post-transplant in NSG mice. Scale bars, 500μm.

(F) H&E (left) and Toluidine Blue (right) staining of subcutaneous transplants of digested day 35 chondrospheroid cells attached to HyA-FMBs at 2 months-post-transplant in NSG mice. High magnification insets shown below their corresponding images. Scale bars, 500μm. Asterisks (*) indicate HyA-FMBs.

(G) Immunofluorescence analyses of COL10A1 and COL2A1 from digested day 35 chondrospheroid cells attached to HyA-FMBs at 2 months-post-transplant in NSG mice. Scale bars, 500μm.

(H) Confocal images of pSMAD5 (red) with nuclei counterstained with DAPI (blue). High magnification image shown to the right outlined by yellow dashed lines. Scale bars, 300μm.
(I) Quantification of nuclear pSMAD5+ staining compared to all DAPI+ cells. ***$p<0.001$ using unpaired two-tailed t-test.

Figure S7, Related to Figure 7. Chondral transplantation of hiPSC/HyA-FMB constructs yields stable chondrogenesis.

(A-B) Undigested chondrospheroid tissues (without HyA-FMBs) were transplanted directly into a chondral defect in immunocompromised NSG mice, followed by tissue histology showing Toluidine Blue (A) and H&E staining (B) at 1 month-post-transplant. Scale bars, 500μm.

(C) H&E staining of defect areas from transplanted day 35 chondrospheroids attached to HyA-FMBs at 1-5 months-post-transplant in NSG mice. High magnification insets shown to the right of their corresponding images. Arrows depicting the formation of bone in control transplants at 1 month. Scale bars, 500μm. Asterisks (*) indicate HyA-FMBs.

(D) Immunofluorescence analysis of human VIMENTIN to confirm human origin of transplanted day 35 chondrospheroids attached to HyA-FMBs at 1 month-post-transplant. Dashed line indicates boundary separating transplanted cells from mouse bone marrow (BM). Nuclei counterstained with DAPI. Scale bar, 100μm. Asterisks (*) indicate HyA-FMBs.

(E) Immunofluorescence analysis of COL1A1 in femoral defects from NSG mice 1 month-post-transplant of digested day 35 chondrospheroids attached to HyA-FMBs. High magnification insets depict transplanted cells in the core (E') and at the edge (E") of the transplant near subchondral bone. Nuclei counterstained with DAPI. Scale bar, 200μm.

(F) H&E staining of defect areas from transplanted day 35 chondrospheroids attached to HyA-FMBs at 2 months-post-transplant in SRG rats (right). Left images depict control transplants, including HyA-FMBs only and hBMSCs/SSCs attached to HyA-FMBs at 2 months. High magnification insets shown to the right of their corresponding images. Scale bars, 500μm. Asterisks (*) indicate HyA-FMBs.

(A,D,E) Orange dashed lines indicate area of transplanted tissue.

(B,C,F) Black dashed lines indicate area of transplanted tissue.

Table S1, related to Figure 4G-I, Figure S3E-F, Figure 7B,G, Figure S7B,D-F

Cartilage grading of hBMSC/SSC and iPSC-derived chondrospheroid transplants

Experimental condition	Transplant site	Recipient	Harvest time (months)	Cartilage score (mean±SD)	Bone score (mean±SD)	#
Sham	Joint	SRG rat	2	0.2 ± 0.4	1.8 ± 0.7	5
HyA-FMB only	Joint	SRG rat	2	0.6 ± 0.5	1.2 ± 0.4	5
hBMSC/SSC + HyA-FMB	Joint	SRG rat	2	1.8 ± 0.4**	3.0 ± 0.8**	6
hBMSC/SSC organoid + HyA-FMB	Joint	SRG rat	2	1.1 ± 0.6	1.9 ± 0.6	7
Sham	Joint	SRG rat	4	0.2 ± 0.4	2.0 ± 0.6	5
HyA-FMB only	Joint	SRG rat	4	0.4 ± 0.5	0.6 ± 0.8*	5
hBMSC/SSC + HyA-FMB	Joint	SRG rat	4	1.2 ± 0.7	3.4 ± 0.5***	5
hBMSC/SSC organoid + HyA-FMB	Joint	SRG rat	4	0.6 ± 0.8	3.2 ± 0.8**	5
Undigested chondrospheroids	Ectopic	NSG mice	2	3.0 ± 0.0	0.2 ± 0.4	3
Digested chondrospheroid cells + Matrigel	Ectopic	NSG mice	2	3.7 ± 0.5	0.0 ± 0.0	3
Digested chondrospheroid cells + HyA-FMBs	Ectopic	NSG mice	2	4.0 ± 0.0	0.0 ± 0.0	3
HyA-FMB only	Joint	NSG mice	1	0.7 ± 0.7	1.4 ± 0.5	6
Digested chondrospheroid cells + HyA-FMBs	Joint	NSG mice	1	3.4 ± 0.5***	0.0 ± 0.0***	8
Digested chondrospheroid cells + HyA-FMBs	Joint	NSG mice	2	4.0 ± 0.0***	0.0 ± 0.0***	6
Digested chondrospheroid cells + HyA-FMBs	Joint	NSG mice	3	4.0 ± 0.0***	0.0 ± 0.0***	4
Digested chondrospheroid cells + HyA-FMBs	Joint	NSG mice	4	4.0 ± 0.0***	0.0 ± 0.0***	4
Digested chondrospheroid cells + HyA-FMBs	Joint	NSG mice	5	4.0 ± 0.0***	0.0 ± 0.0***	3
HyA-FMB only	Joint	SRG rats	2	0.6 ± 0.5	1.2 ± 0.4	5
Digested chondrospheroid cells + HyA-FMBs	Joint	SRG rats	2	3.7 ± 0.5***	0.0 ± 0.0***	3
Digested chondrospheroid cells + HyA-FMBs	Joint	SRG rats	5	4.0 ± 0.0***	0.0 ± 0.0***	3

Unpaired two-tailed t tests were used for comparison of experimental groups with HyA-FMB only controls: *p<0.05, **p<0.01, ***p<0.001.

Table S5, related to STAR*METHODS – Human bone marrow stromal cell/ skeletal stem cell (hBMSC/SSC) isolation and culture

hBMSC/SSC donors

Donor	Age	Sex	Part of skeleton	Diagnosis	Experiment
1	14	Female	Right hip	Hip dislocation/spasticity	scRNA-seq
2	18	Male	Femur	Myopathy	scRNA-seq
3	8	Male	Femur	Cerebral palsy	scRNA-seq
4	4	Female	Femur	Sickle cell anemia	Transplant

Table S6, related to STAR*METHODS - PCR and RNA sequencing

Oligonucleotide sequences used for qRT-PCR (human)

Gene	Forward (5'-3')	Reverse (5'-3')	GenBank
OCT4	AGTGAGAGGCAACCTGGA	ACACTCGGACCACATCCTT	NM_002701.6
MIXL1	GGTACCCCGACATCCACT	TAATCTCCGGCCTAGCCAA	NM_031944.3
BRACHY	TGCTTCCCTGAGACCCAG	GATCACTTCTTTCCTTTGCA	NM_003181.4
GSC	GAGGAGAAAGTGGAGGT	CTCTGATGAGGACCGCTTC	NM_173849.3
EOMES	CAACATAAACGGACTCAAT	ACCACCTCTACGAACACAT	NM_005442.4
FOXA2	GGGAGCGGTGAAGATGG	TCATGTTGCTCACGGAGGA	NM_021784.5
MESP1	GAAGTGGTTCCTTGGCAG	TCCTGCTTGCCTCAAAGTG	NM_018670.4
MESP2	AGCTTGGGTGCCTCCTTA	TGCTTCCCTGAAAGACATC	NM_001039958.
FOXF1	AGCAGCCGTATCTGCACC	CTCCTTTCGGTCACACATG	NM_001451.3
TBX6	AAGTACCAACCCCGCATA	TAGGCTGTCACGGAGATGA	NM_004608.4
CDX2	GGGCTCTCTGAGAGGCA	CCTTTGCTCTGCGGTTCTG	NM_001265.6
MSGN1	CGGAATTACCTGCCACCT	GGTCTGTGAGTTCCCCGAT	NM_001105569.
DLL1	ACTCCGCGTTCAGCAACC	TGGGTTTTCTGTTGCGAGG	NM_005618.4
FOXC2	CCTCCTGGTATCTCAACCA	GAGGGTCGAGTTCTCAATC	NM_005251.3
PARAXIS	GAGCTGAGGAGAGTCCC	TGTGCCTCTCTCTAGGTCC	KT583946.1
MEOX1	TCTGAGCGCCAGGTCAAA	CTGAACTTGGAGAGGCTGT	NM_001040002.
BAPX1	GATTTCAGGCCTGCTGGG	TTTCGCACCCCTTGGTTAC	NM_001189.4
NKX3.1	CCAGCTCAGGTGACAACC	CTTGGCCCCTTGTGCTTTT	NM_006167.4
PAX3	CTCCACGCTCCGGATAGT	ATCTTGTGGCGGATGTGGT	NM_181457.4
PAX9	TGGTTATGTTGCTGGACAT	GGAAGCCGTGACAGAATG	NM_001372076.
HAND1	GTGCGTCCTTTAATCCTCT	GTGAGAGCAAGCGGAAAA	NM_004821.3
ISL1	AGATTATATCAGGTTGTAC	ACACAGCGGAAACACTCG	NM_002202.3
TBX5	TACCACCACACCCATCAA	ACACCAAGACAGGGACAG	NM_181486.4
PRRX1	TGATGCTTTTGTGCGAGA	AGGGAAGCGTTTTTATTGG	NM_006902.5
HOXB5	AACTCCTTCTCGGGGCGT	CATCCCATTGTAATTGTAGC	NM_002147.4
TBX20	GGCGACGGAGAACACAAT	CTGGGCACAGGACGACTT	NM_001166220.
NKX2.5	CAAGTGTGCGTCTGCCTT	CAGCTCTTTCTTTTCGGCT	NM_001166176.
GAPDH	ATGGGGAAGGTGAAGGTC	TAAAAGCAGCCCTGGTGAC	NM_002046.7