PNAS

Supporting Information for
Modeling human skeletal development using human pluripotent stem cells

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This PDF file includes:

Supporting methods

Quantitative RT-PCR

RNA was extracted from monolayer cultures using TRIzol (ThermoFisher Scientific) following the manufacturer's instructions. Typically, 100-200ng total RNA was used for cDNA synthesis in a total volume of 20µl using the QuantiTect® Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. Quantitative RT-PCR (qPCR) was performed in triplicate with the Brilliant III Ultra-Fast SYBR Green QRT-PCR Kit (Agilent) using 10 µl reactions consisting of 1× Brilliant III Ultra-Fast SYBR Green QRT-PCR Master Mix, 1 µl cDNA, and 1 µM each primer. Amplification was performed in a 384 well plate format on the LightCycler 480 Instrument II (Roche Life Sciences), using a thermocycling protocol involving 40 cycles of denaturation at 95°C for 20 seconds, annealing at 55°C for 20 seconds, and amplification at 72°C for 20 seconds. Gene-specific primers were designed using Primer-BLAST (NCBI) (Table S1). To avoid amplification from genomic DNA, primer sets were designed to span multiple exons and PCR products analyzed by agarose gel electrophoresis to confirm single products corresponding to the predicted cDNA amplicons for each gene. Analyses used LightCycler 480 Software (release 1.5.1.62). Human β-actin (*ACTB*) was routinely used as a house-keeping reference gene. Graphs were drawn using GraphPad Prism 9.

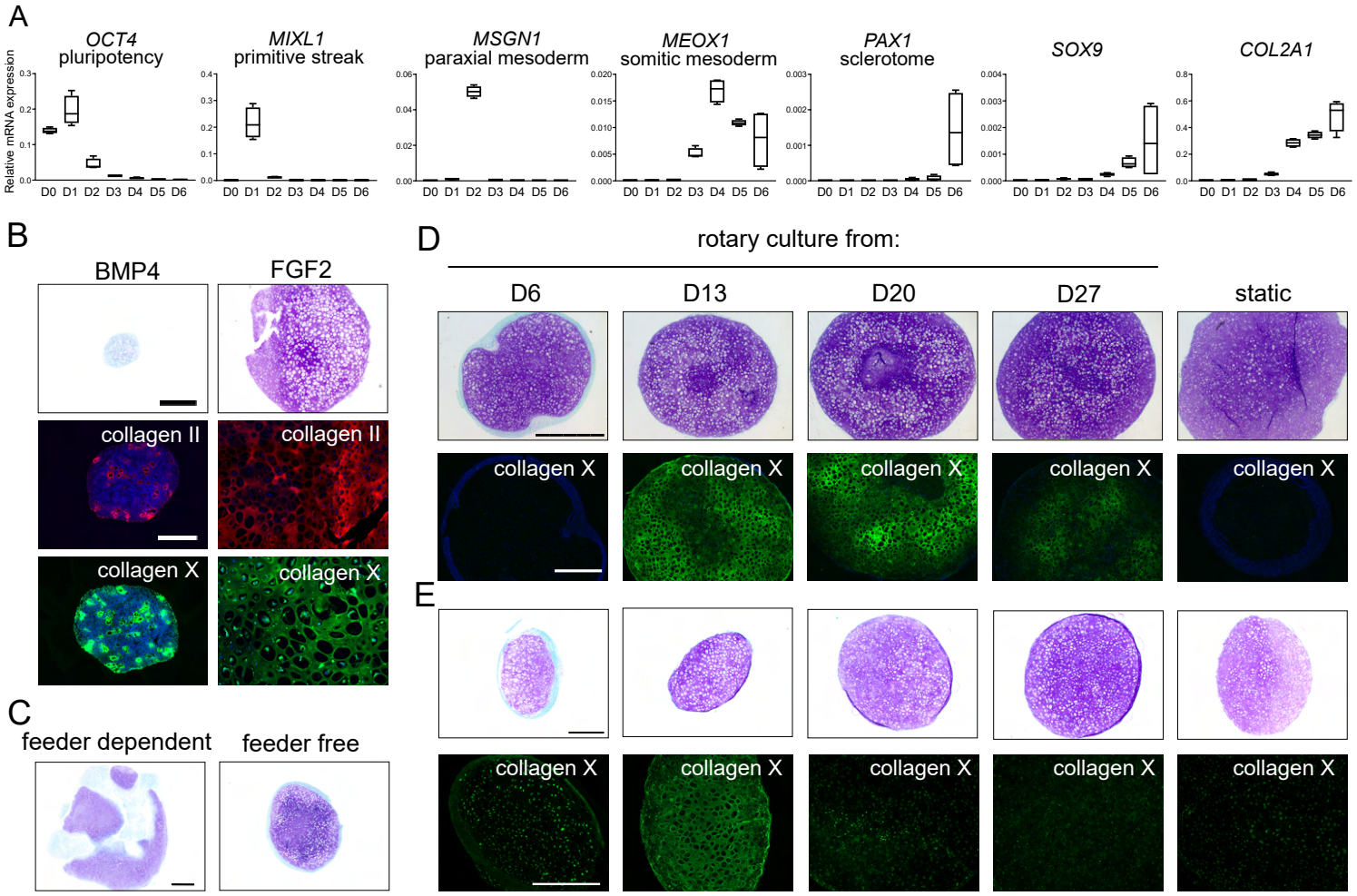
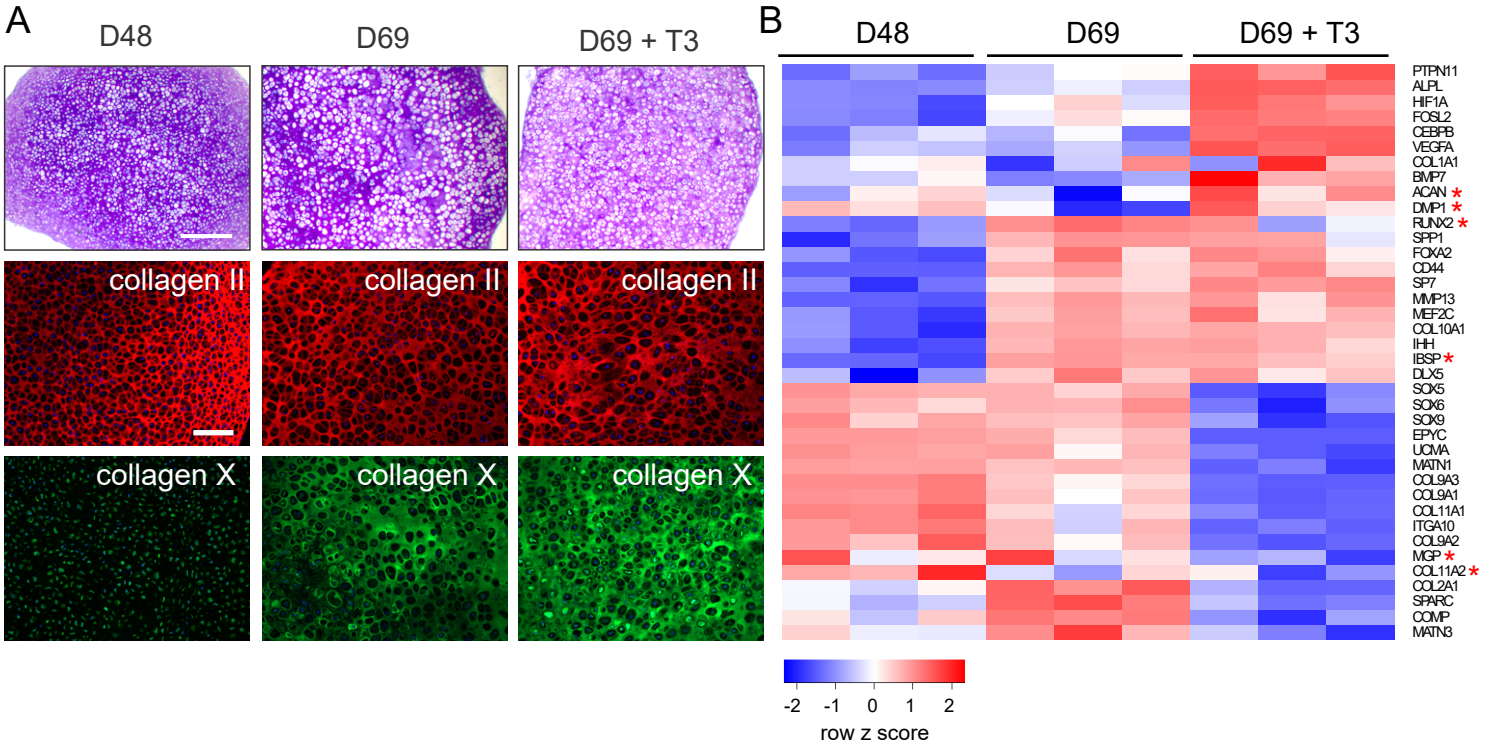
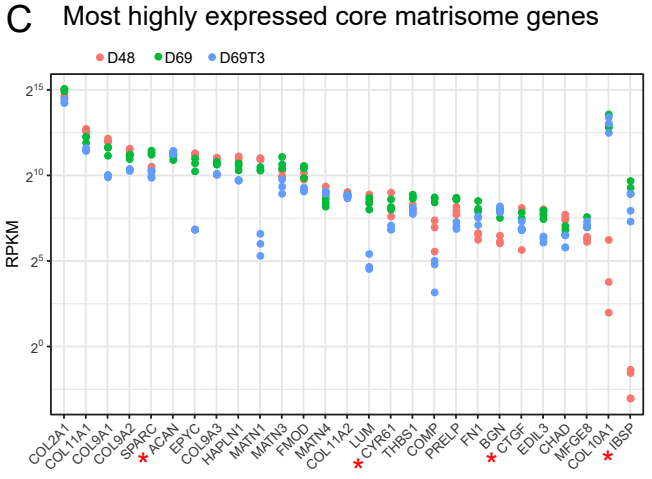
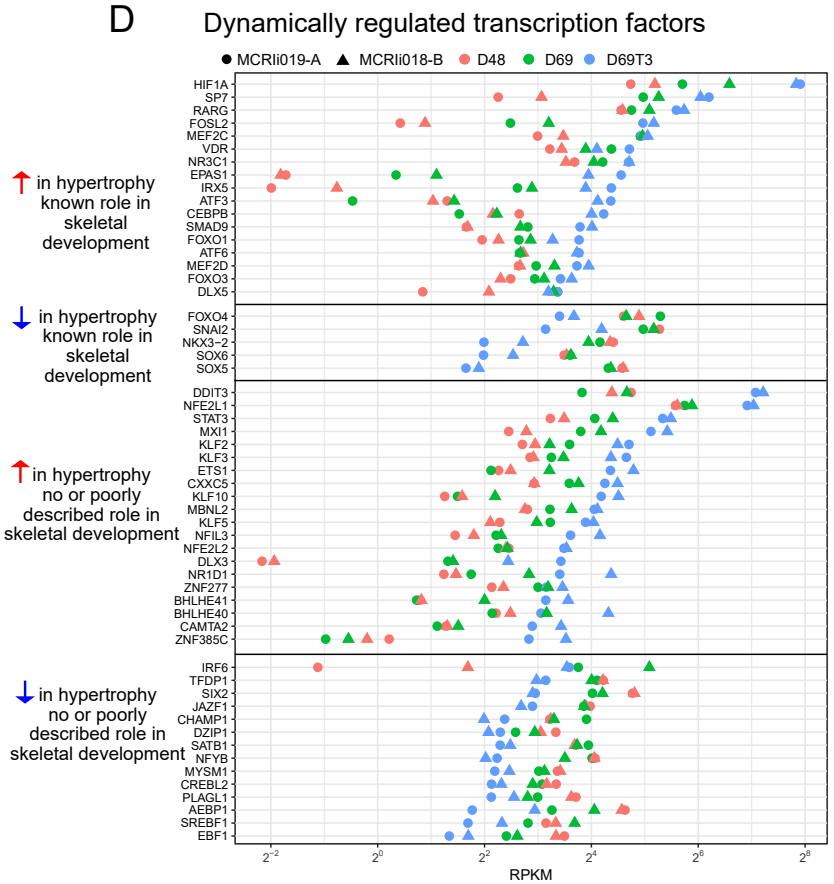
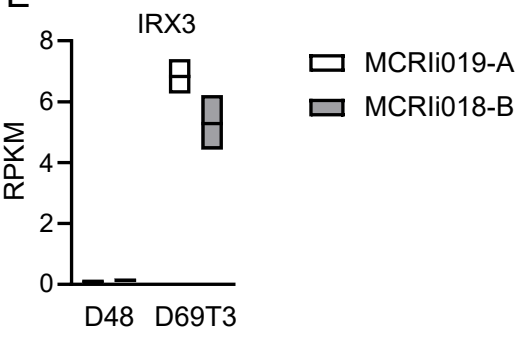

A
OCT4
pluripotency
MIXL1
primitive streak
MSGN1
paraxial mesoderm
MEOX1
somitic mesoderm
PAX1
sclerotome
SOX9
COL2A1
Relative mRNA expression
D0 D1 D2 D3 D4 D5 D6
B
BMP4
FGF2
collagen II
collagen X
C
feeder dependent
feeder free
D
rotary culture from:
D6
D13
D20
D27
static
collagen X
E
collagen X
F
D48
-TGFβ3
D48
+TGFβ3
collagen II
collagen X
PRG4
G
core matrisome
most highly expressed +TGFβ
- TGFβ
+ TGFβ
COL2A1
SPARC
COL11A1
FMOD
COL3A1
PRG4
COL9A1
COL1A1
POSTN
COL1A2
COL11A2
COL9A2
HAPLN1
FN1
COMP
BGN
ACAN
COL9A3
ASPN
MFAP4
RPKM
H
core matrisome
most differentially expressed +TGFβ
PRG4
COL1A1
COL1A2
POSTN
COL11A2
COL9A2
ACAN
HAPLN1
ASPN
COL5A2
OGN
EPYC
ELN
ABI3BP
LUM
TGFBI
MATN3
LTBP2
SPP1
PCOLCE
TNC
MFGE8
CRISPLD1
MFAP5
NID2
HSPG2
COL22A1
MATN1
KERA
CILP
PCOLCE2
NID1
SPARCL1
OMD
CRISPLD2
DPT
COL10A1
PAPLN
IBSP
EFEMP1
average RPKM D48 +TGFβ
logFC
I
transcription factors
most highly expressed +TGFβ
- TGFβ
+ TGFβ
AEBP1
YBX1
SOX9
ATF4
CREB3L2
HIF1A
NFE2L1
TSC22D1
TRPS1
ELK3
HMGN3
XBP1
ERF
MKX
ERG
MAZ
NFIX
MEF2A
PLAGL1
BHLHE40
RPKM
J
transcription factors
most differentially expressed +TGFβ
AEBP1
SOX9
ZNF521
ERG
MEOX2
TRPS1
MKX
ELK3
NFAT5
SIX2
BHLHE40
KLF10
CREB3L1
JUNB
NCOA2
MEF2C
ZEB1
SKI
PRRX1
SNAI1
ATF5
SATB1
BARX1
FOXO4
TCF4
CHAMP1
GLIS2
ZNF385B
SOX6
THRB
NFATC2
HMGA1
MYC
SCX
MXI1
RUNX2
KLF5
SP7
DLX5
IRF6
average RPKM D48 +TGFβ
logFC

Fig. S1. (*A*) Gene expression of stage specific markers during the 6-day differentiation to sclerotome in iPSC line MCRIi019-A. N = 4 parallel differentiations. (*B*) The iPSC line MCRIi018-B was differentiated to sclerotome with cell pellets formed at day 4 and retained in static pellet culture throughout. At the end of day 6, pellets were supplemented with either 20 ng/ml BMP4 or 20 ng/ml FGF2 for 14 days. Toluidine blue (scale bar is 500 µm) and collagen II and X immunostained pellet sections (scale bar is 200 µm) at day 48. (*C*) MCRIi001-A-2 was adapted to feeder free conditions, then feeder-dependent and feeder-free versions differentiated to chondrocytes with pellets transferred to rotary culture at the end of day 6. Toluidine blue stained chondronoids at day 62. Scale bar is 500 µm. (*D*, *E*) Rotary culture influences chondrocyte maturity. The iPSC lines MCRIi001-A-2 (*D*) and MCRIi019-A (*E*) were differentiated to sclerotome with cell pellets formed at day 4. At the end of day 6, pellets were supplemented with 20 ng/ml FGF2 for 14 days. Some pellets were transferred to rotary culture at the end of day 6, day 13, day 20 or day 27 and some remained in static culture. Toluidine blue and collagen X immunostained pellets at day 48. Scale bars are 500 µm. (*F*) TGFβ3 induces an articular chondrocyte phenotype. The iPSC line MCRIi019-A was differentiated to sclerotome with cell pellets formed at day 4. At the end of day 6 all sclerotome pellets were supplemented with 20 ng/ml FGF2 for 14 days and some pellets were treated with 10 ng/ml TGFβ3 from day 13. Pellets were transferred to rotary culture at day 20. Histology and collagen II, collagen X and PRG4 immunostaining at day 48. Scale bars are 500 µm. (*G*) mRNA abundance (log_2 RPKM) of the 20 core matrisome components most highly expressed at day 48 in TGFβ3 treated chondronoids. Abundance in untreated chondronoids is shown for comparison. (*H*) mRNA abundance at day 48 in TGFβ3 treated chondronoids (average log_2 RPKM) of the 20 most upregulated and 20 most downregulated core matrisome genes (logFC, adj.P.value < 0.05, average RPKM >10 in at least one treatment group). (*I*) mRNA abundance (log_2 RPKM) of the 20 transcription factors most highly expressed at day 48 in TGFβ3 treated chondronoids. Abundance in untreated chondronoids is shown for comparison. (*J*) mRNA abundance at day 48 in TGFβ3 treated chondronoids (average log_2 RPKM) of the 20 most upregulated and 20 most downregulated transcription factor genes (logFC, adj.P.value < 0.05, average RPKM >10 in at least one treatment group). RNAseq data N = 4 parallel differentiations.


A
D48
D69
D69 + T3
collagen II
collagen X
B
D48
D69
D69 + T3
PTPN11
ALPL
HIF1A
FOSL2
CEBPB
VEGFA
COL1A1
BMP7
ACAN *
DMP1 *
RUNX2 *
SPP1
FOXA2
CD44
SP7
MMP13
MEF2C
COL10A1
IHH
IBSP *
DLX5
SOX5
SOX6
SOX9
EPYC
UCMA
MATN1
COL9A3
COL9A1
COL11A1
ITGA10
COL9A2
MGP *
COL11A2 *
COL2A1
SPARC
COMP
MATN3
-2 -1 0 1 2
row z score


C
Most highly expressed core matrisome genes
D48 D69 D69T3
RPKM
COL2A1
COL11A1
COL9A1
COL9A2
SPARC
ACAN
EPYC
COL9A3
HAPLN1
MATN1
MATN3
FMOD
MATN4
COL11A2
LUM
CYR61
THBS1
COMP
PRELP
FN1
BGN
CTGF
EDIL3
CHAD
MFGE8
COL10A1
IBSP


D
Dynamically regulated transcription factors
MCRIi019-A MCRIi018-B D48 D69 D69T3
HIF1A
SP7
RARG
FOSL2
MEF2C
VDR
NR3C1
EPAS1
IRX5
ATF3
CEBPB
SMAD9
FOXO1
ATF6
MEF2D
FOXO3
DLX5
FOXO4
SNAI2
NKX3−2
SOX6
SOX5
DDIT3
NFE2L1
STAT3
MXI1
KLF2
KLF3
ETS1
CXXC5
KLF10
MBNL2
KLF5
NFIL3
NFE2L2
DLX3
NR1D1
ZNF277
BHLHE41
BHLHE40
CAMTA2
ZNF385C
IRF6
TFDP1
SIX2
JAZF1
CHAMP1
DZIP1
SATB1
NFYB
MYSM1
CREBL2
PLAGL1
AEBP1
SREBF1
EBF1
RPKM
in hypertrophy known role in skeletal development
in hypertrophy known role in skeletal development
in hypertrophy no or poorly described role in skeletal development
in hypertrophy no or poorly described role in skeletal development


E
IRX3
RPKM
MCRIi019-A
MCRIi018-B
D48 D69T3

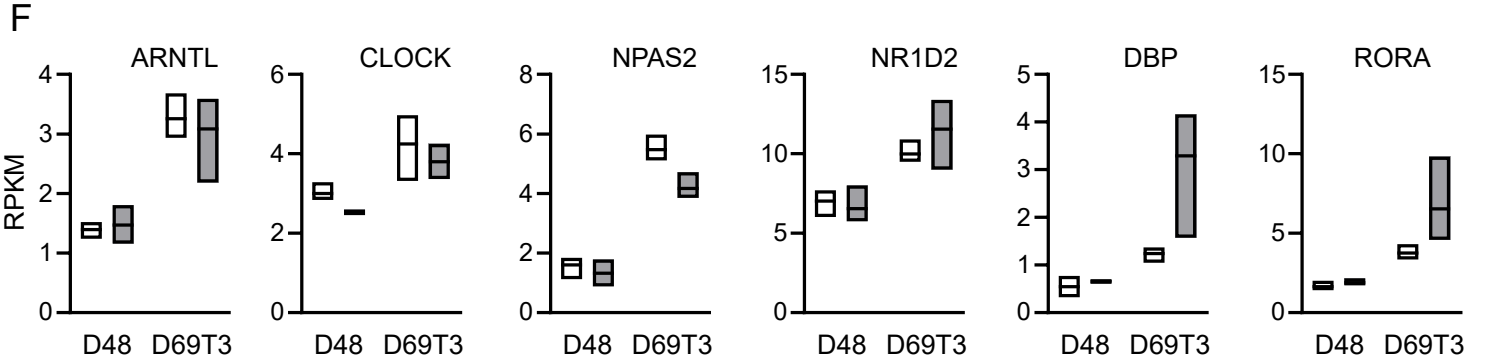
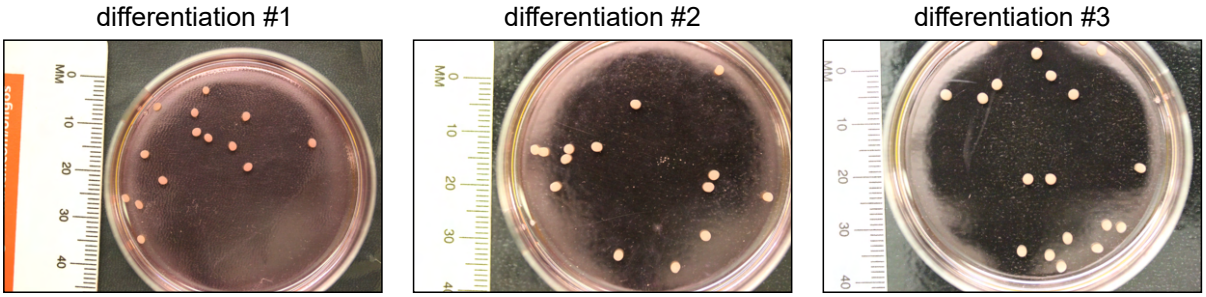

F
ARNTL
CLOCK
NPAS2
NR1D2
DBP
RORA
RPKM
D48 D69T3

Fig. S2. Reproducible differentiation to hypertrophic cartilage in a second iPSC line. MCRIi018-B was differentiated to chondrocytes then some organoids were treated with T3 for 3 weeks from D48 to D69. (*A*) Toluidine blue and collagen II and X immunostaining showing chondrocytes mature to hypertrophy. (*B*) Changes in mRNA abundance for selected cartilage, hypertrophic cartilage and bone proteins. N = 3 independent differentiation experiments. (*C*) The most highly expressed core matrisome genes. Each point represents a determination in three independent differentiations. Highly expressed genes not reaching the statistical threshold for differential expression (adj.P.value < 0.05) are indicated with a red asterisk. (*D*) Transcription factors dynamically regulated during maturation to hypertrophy. Transcription factors differentially expressed in the RNAseq D69T3 v D48 comparison (adj.P.value ≤ 0.05, LogFC ≥ 1 or ≤ -1) and expressed at average RPKM >10 at one or more cell line/day/treatment are shown grouped by the direction of change and their known or poorly described role in cartilage development. The points show the average $\log_2$ RPKM for each group, N = 3 independent differentiations. (*E*) *IRX3* expression. *IRX3* mRNA abundance fell below the cutoff for panel (*D*). (*F*) mRNA abundance of transcription factors in the KEGG circadian rhythm gene set that are dynamically regulated and not shown in panel (*D*).

A MCRIi018-B at D34



B

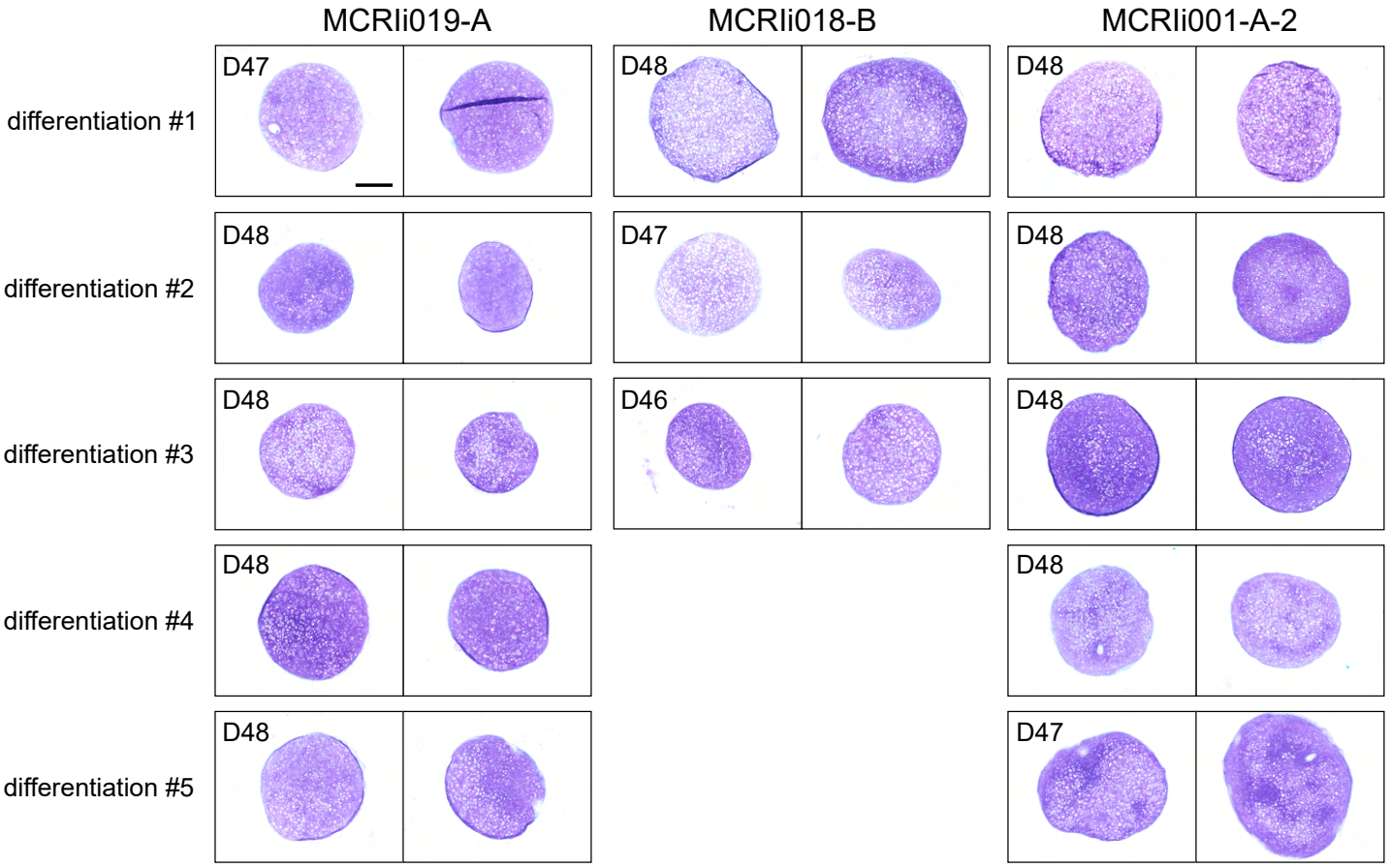


Fig. S3. Reproducible iPSC to chondrocyte differentiation. (*A*) Images show MCRIi018-B chondronoids in 6 cm non-adherent dishes at D34 in three independent differentiations. Note the consistent chondronoid size within and between independent differentiations. (*B*) Replicate toluidine blue stained chondronoids from 3-5 independent differentiations in three iPSC lines, MCRIi019-A, MCRIi018-B and MCRIi001-A-2. The analysis day, D46, 47 or 48, is indicated for each differentiation. In all lines in all differentiations there is uniform cartilage tissue with no indication of other cell types. Scale bar is 500 µm. Note that the sections are not always at the centre of the chondronoid so they do not reflect the actual chondronoid size.

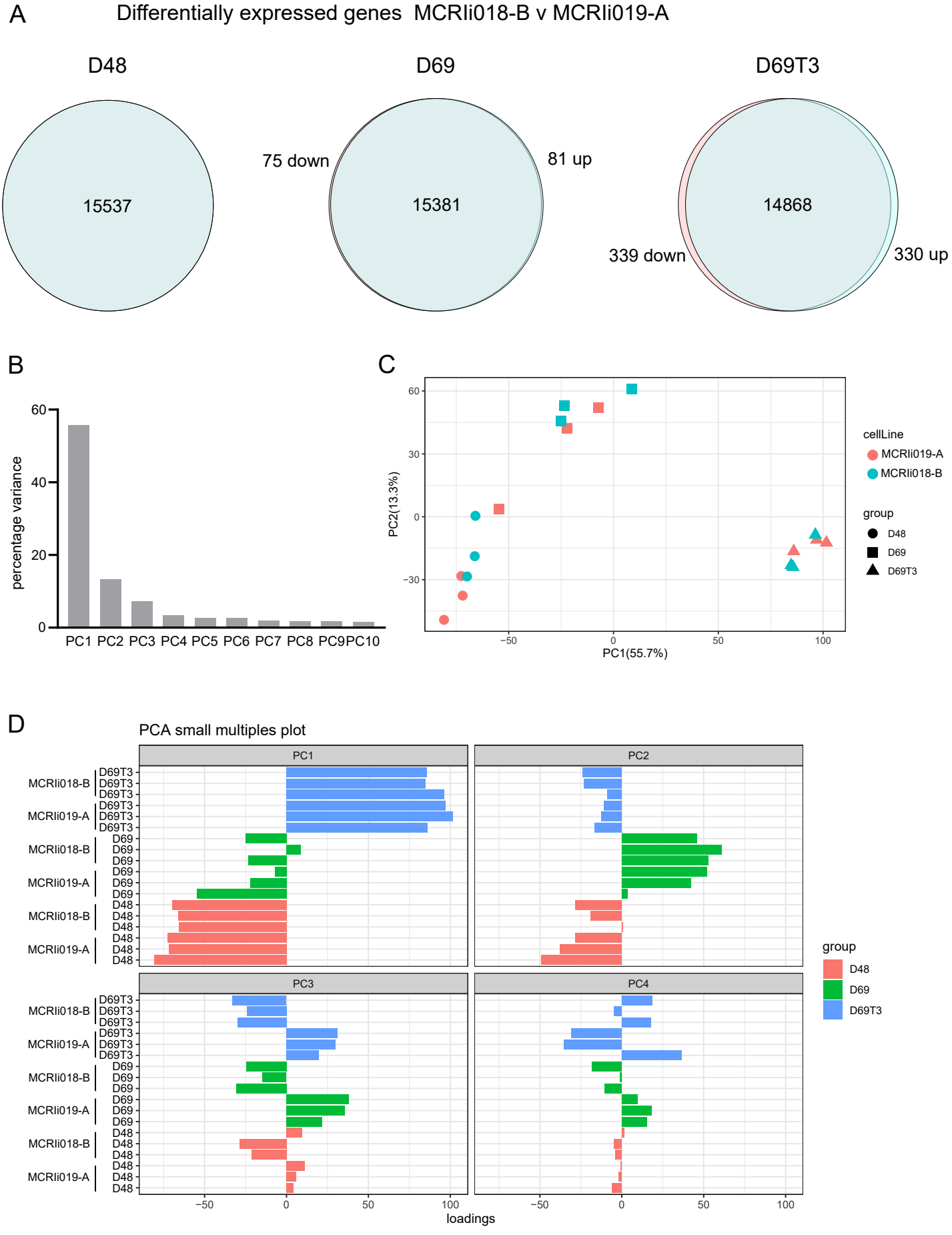
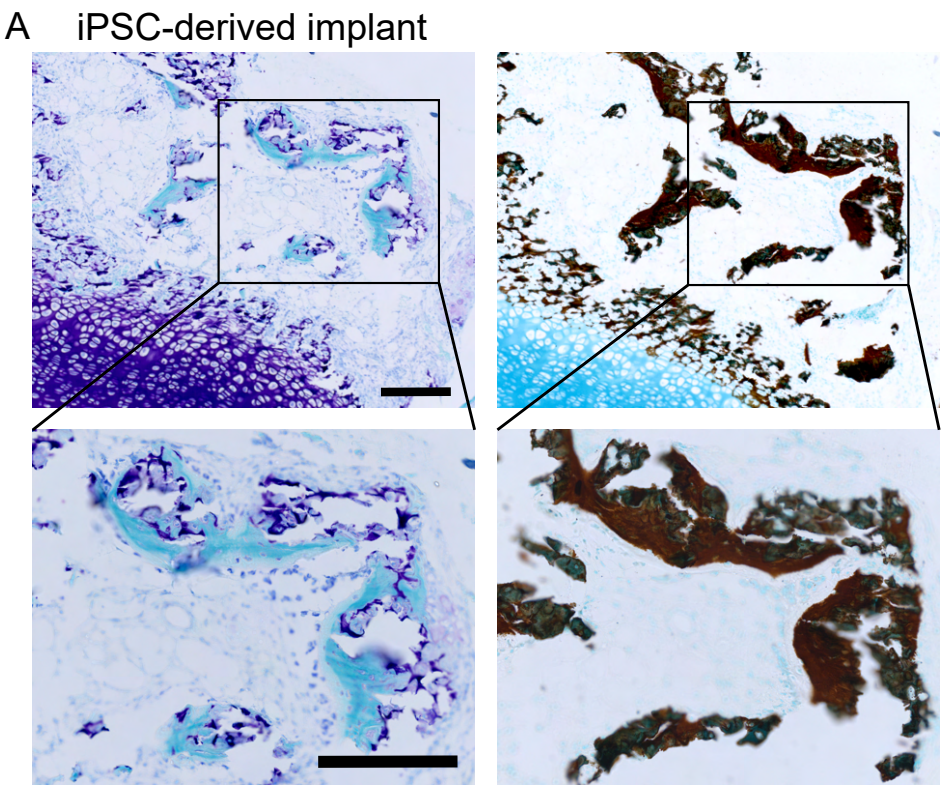
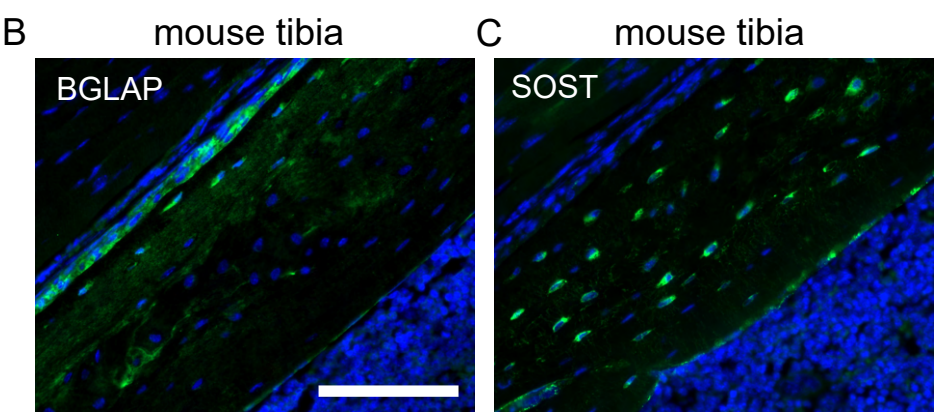

A
Differentially expressed genes MCRIi018-B v MCRIi019-A
D48
D69
D69T3
15537
75 down
15381
81 up
14868
339 down
330 up
B
percentage variance
PC1 PC2 PC3 PC4 PC5 PC6 PC7 PC8 PC9 PC10
C
PC2(13.3%)
PC1(55.7%)
cellLine
MCRIi019-A
MCRIi018-B
group
D48
D69
D69T3
D
PCA small multiples plot
PC1
PC2
PC3
PC4
MCRIi018-B
MCRIi019-A
loadings
group
D48
D69
D69T3

Fig. S4. Reproducible gene expression profiles in iPSC-derived chondrocyte maturation. The data relate to the differentiation experiments presented in Fig. 1B-E and Fig. S2. There were three independent differentiations of two iPSC lines, MCRIi018-B and MCRIi019-A. RNA was isolated from all samples at the same time and the RNAseq libraries were made at the same time using the same batch of reagents. (*A*) The number of differentially expressed genes (adj.P.val ≤ 0.05) at D48, D69 and D69 following 3 weeks supplementation with T3. There were no differentially expressed genes at D48. (*B*) The percentage variance explained by each principal component. (*C*) Principal component analysis (PCA) plot, PC1 v PC2, showing that samples from independent differentiations of two iPSC lines cluster closely and separate based on differentiation day and treatment. (*D*) Small multiples plot emphasising that PC1 and PC2, together accounting for 69% of the variation, are largely cell line and differentiation independent. It is only when looking at PC3, which accounts for only 7.3% of the variability that the samples can be separated by iPSC line. PCA was done using the function prcomp in base r.


A
iPSC-derived implant


B
mouse tibia
BGLAP
C
mouse tibia
SOST

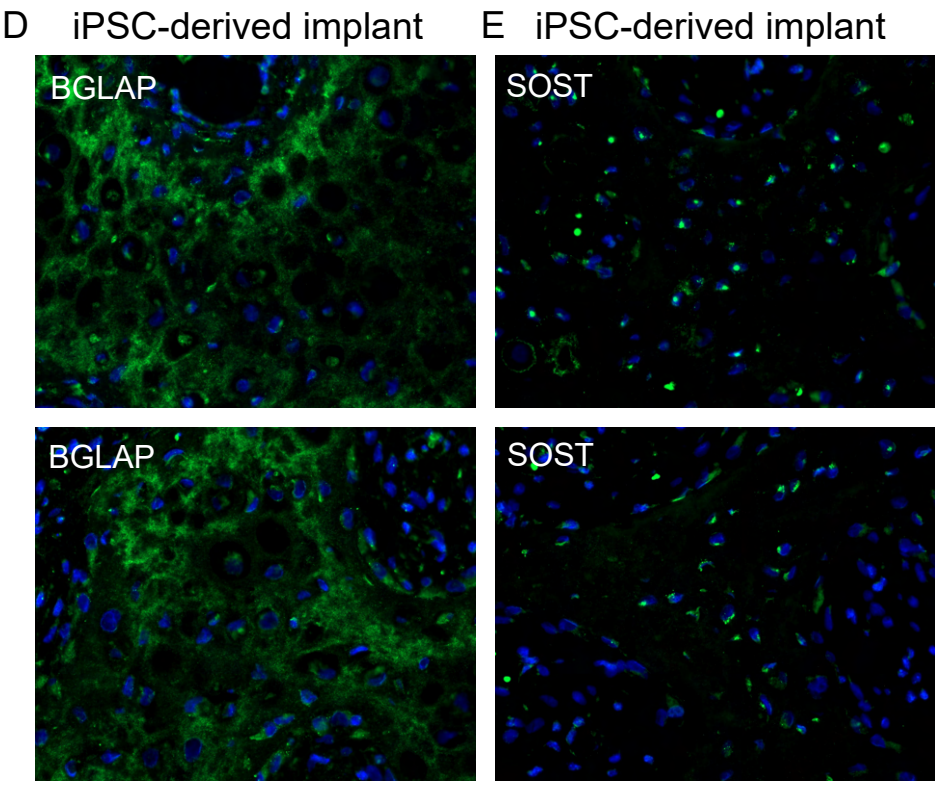

D
iPSC-derived implant
BGLAP
BGLAP
E
iPSC-derived implant
SOST
SOST

Fig. S5. Hypertrophic chondrocyte to osteoblast/osteocyte transition in iPSC-derived organoid implants. (*A*) Hypertrophic chondronoids were implanted subcutaneously into immunocompromised mice and harvested after 13 weeks. Implants were sectioned and stained with toluidine blue for cartilage proteoglycans and fast green to highlight bone. The image on the right is an adjacent section stained with methyl green for cartilage and von Kossa for mineralization. Higher power images of the areas indicated with the boxes are shown below. The mineralized areas correspond to the fast green positive bone. Scale bars are 200 μm. (*B*) BGLAP immunostaining in 4-week-old mouse tibia cortical bone. (*C*) SOST immunostaining in 4-week-old mouse tibia cortical bone. (*D*) BGLAP immunostaining in a decalcified iPSC-derived implant. (*E*) SOST immunostaining in a decalcified iPSC-derived implant. The expression and distribution of BGLAP and SOST are similar in the implant bone and mouse bone. Images in panels B-E were taken at the same magnification. Scale bar is 100 μm.

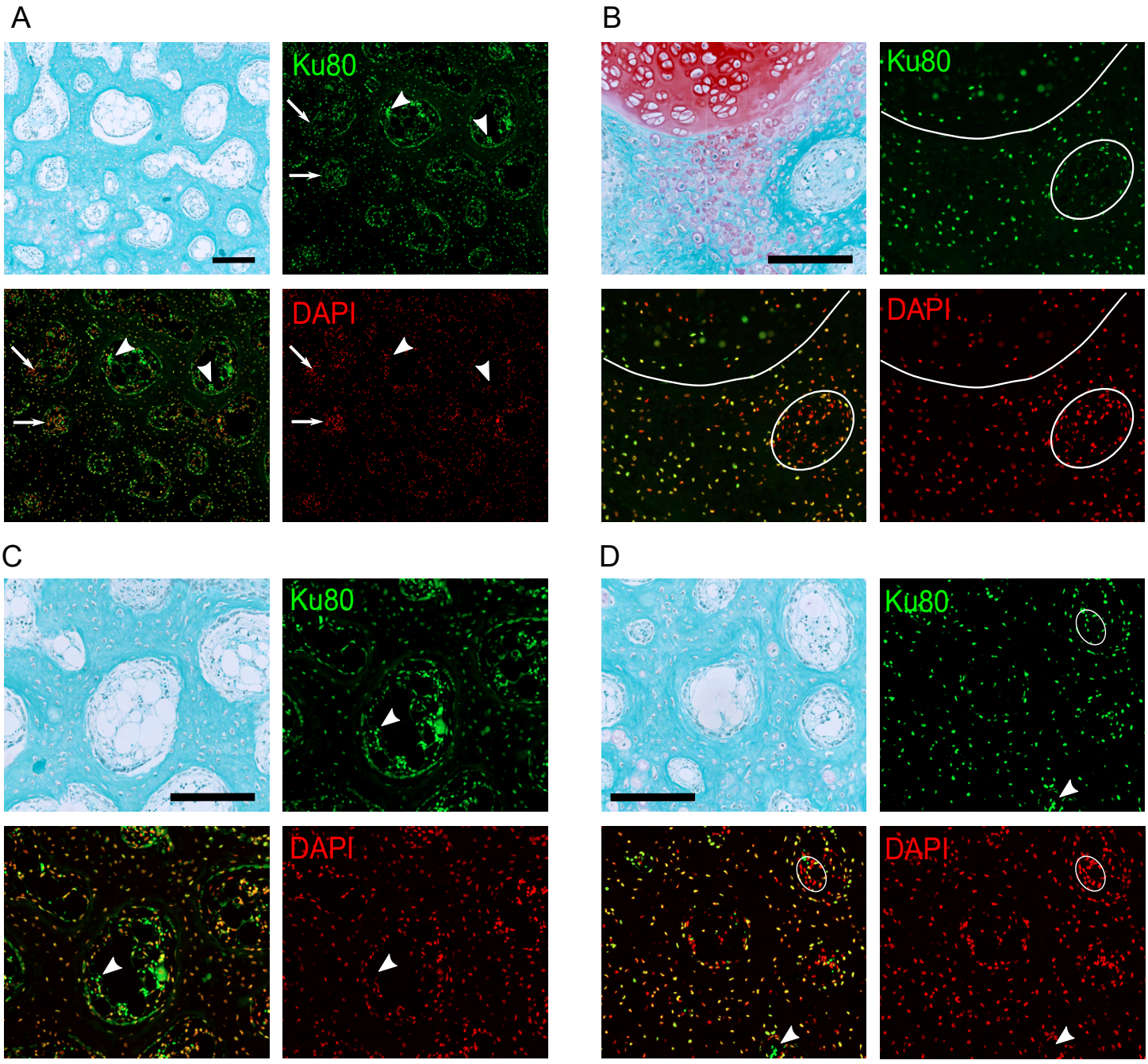

A
Ku80
DAPI
B
Ku80
DAPI
C
Ku80
DAPI
D
Ku80
DAPI

Fig. S6. Osteocytes in iPSC-derived organoid implants are human. Implants were decalcified, sectioned, and stained with safranin O for cartilage proteoglycans and fast green to highlight bone. Adjacent sections were immunostained with the human specific antibody Ku80. DAPI staining indicates cell nuclei. There was extensive blood vessel invasion into the implant and large areas of bone surrounding the blood vessels. (*A*) Blood vessels in the implant contain red blood cells that autofluoresce in the green channel and are DAPI negative (arrow heads). Blood vessels also contain mouse cells indicated by red nuclei that do not stain with Ku80 (arrows). Osteocytes in the bone are Ku80 and DAPI positive indicating they are human and derived from the implanted iPSC-cartilage. (*B*) Image shows a cartilage area (safranin O stained) and an adjacent area where the chondrocytes are transitioning to osteoblasts, remodelling the cartilage ECM and depositing bone. The blood vessel (circled) contains human cells (Ku80 and DAPI positive) and mouse cells (Ku80 negative, DAPI positive). All the osteocytes in the bone are both Ku80 and DAPI positive indicating they are human. (*C*) The blood vessel in the center of the image contains autofluorescing red blood cells (arrowhead indicates some examples). Osteocytes in the bone are Ku80 and DAPI positive indicating they are human. (*D*) Mouse cells (DAPI positive, Ku80 negative) in a blood vessel (circled), and autofluorescing mouse red blood cells (DAPI negative) a blood vessel (arrowhead). Osteocytes in the bone are Ku80 and DAPI positive indicating they are human. We found no evidence that there were mouse cells in the bone. All scale bars are 200 μm.

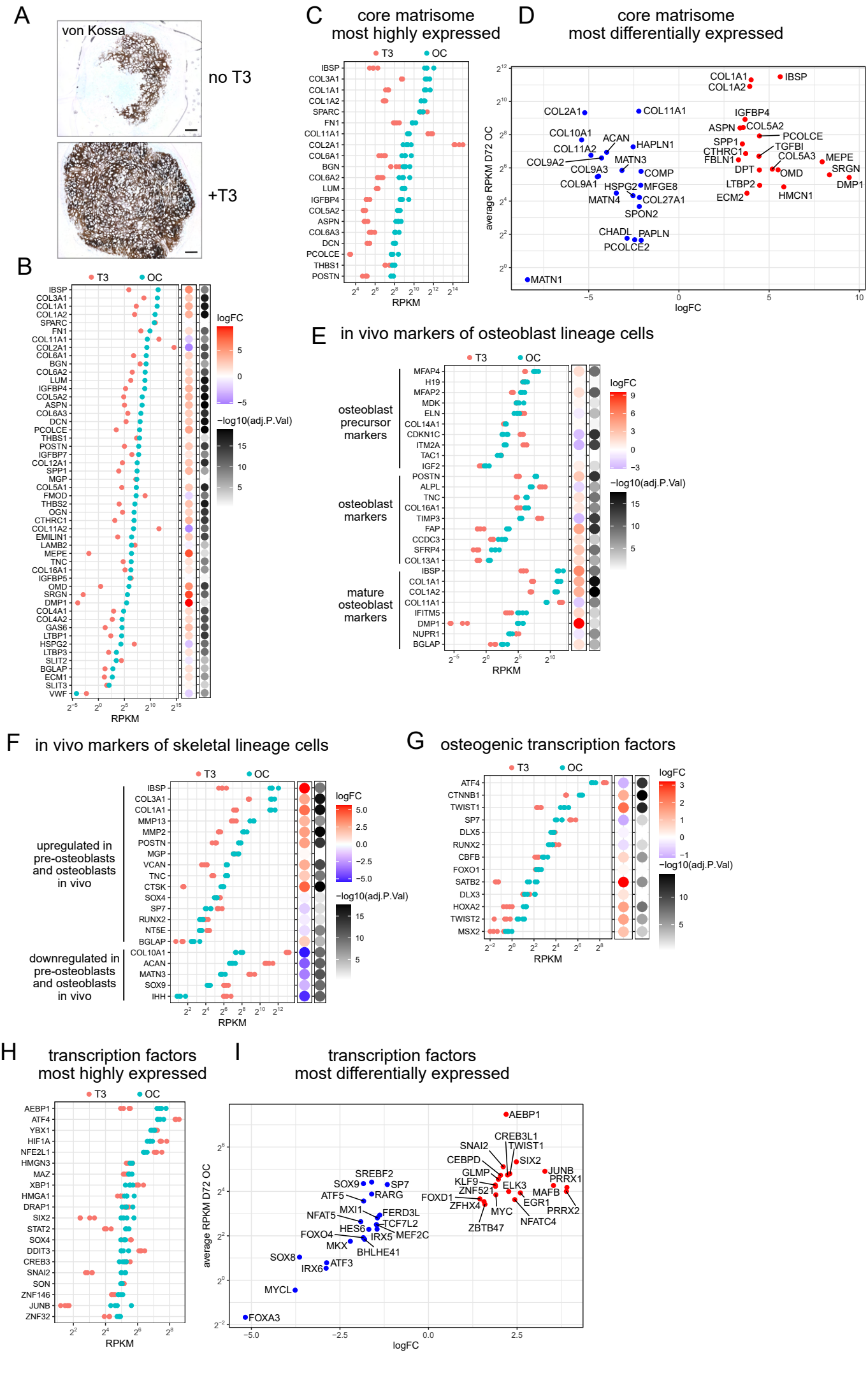

A
von Kossa
no T3
+T3
B
T3
OC
logFC
−log10(adj.P.Val)
RPKM
C
core matrisome
most highly expressed
D
core matrisome
most differentially expressed
average RPKM D72 OC
logFC
E
in vivo markers of osteoblast lineage cells
osteoblast precursor markers
osteoblast markers
mature osteoblast markers
F
in vivo markers of skeletal lineage cells
upregulated in pre-osteoblasts and osteoblasts in vivo
downregulated in pre-osteoblasts and osteoblasts in vivo
G
osteogenic transcription factors
H
transcription factors
most highly expressed
I
transcription factors
most differentially expressed

Fig. S7. Reproducible *in vitro* hypertrophic chondrocyte to osteoblast transition. (*A*) T3 treatment primes for transition to osteoblasts. MCRIi001-A-2 was differentiated to cartilage and allowed to mature until day 68. Some chondronoids were then treated with 10 nM T3 for 14 days. From day 82 transition to osteoblasts was induced with osteogenic medium for 3 weeks. There was more extensive and intense von Kossa staining in organoids that had been pre-treated with T3 compared to those without T3 treatment. Scale bar is 200 µm. (*B*) The relative average expression in MCRIi019-A iPSC-derived hypertrophic cartilage (T3) and osteoblasts (OC) of the 50 most highly expressed core matrisome genes in osteocytes isolated from mouse tibia (1). N = 4 parallel differentiations. (*C*) The most highly expressed core matrisome genes after 3 weeks in osteogenic conditions (OC) (MCRIi019-A) and their relative expression in hypertrophic cartilage (T3). (*D*) The 20 most upregulated and 20 most downregulated core matrisome genes in osteogenic conditions vs hypertrophic cartilage. (*E*) Expression of the top transcripts that correspond to osteoblast precursor, osteoblast and mature osteoblast clusters in scRNAseq of cells isolated from mouse calvaria (2) during *in vitro* transdifferentiation. Bubble plot shows logFC and adj.P.value during *in vitro* transdifferentiation. (*F*) Expression of genes that mark skeletal cell subpopulations in scRNAseq of *in vivo* mouse transdifferentiation (3) during *in vitro* transdifferentiation. (*G*) Expression of transcription factors known to regulate osteoblast differentiation (4) during *in vitro* transdifferentiation. N = 4 parallel differentiations. (*H*) The most highly expressed transcription factor genes after 3 weeks in osteogenic conditions (OC) and their relative expression in hypertrophic cartilage (T3). (I) The 20 most upregulated and 20 most downregulated transcription factor genes in osteogenic conditions vs hypertrophic cartilage.

Table S1. RT-PCR primer sets for monitoring iPSC to sclerotome differentiation.

Gene	Primer	Sequence 5'-3'
OCT4	hOCT4.F	GAAGTGGGTGGAGGAAGCTG
	hOCT4.R	TAGTCGCTGCTTGATCGCTT
MIXL1	hMIXL1.F	GGTACCCCGACATCCACTTG
	hMIXL1.R	GGGCAGGCAGTTCACATCTA
MSGN1	hMSGN1.F	GGCCTGGTAGAGGTGGACTA
	hMSGN1.R	ACAGGTGGCAGGTAATTCCG
MEOX1	hMEOX1.F	ACTCGGCTCCGCAGATATGA
	hMEOX1.R	GAACTTGGAGAGGCTGTGGA
PAX1	hPAX1.F	ACTTCCCTGCCAAAGGTAGC
	hPAX1.R	CCTCACACCTTCAAATGCCC
SOX9	hSOX9.F	AAGTCGGTGAAGAACGGGC
	hSOX9.R	TCTCGCTTCAGGTCAGCCTT
COL2A1	hCOL2A1.F	TCACGTACACTGCCCTGAAG
	hCOL2A1.R	GCCCTATGTCCACACCGAAT
ACTB	hACTB.F	AAGTCCCTTGCCATCCTAAAA
	hACTB.R	ATGCTATCACCTCCCCTGTG

SI References

1. S. E. Youlten, *et al.*, Osteocyte transcriptome mapping identifies a molecular landscape controlling skeletal homeostasis and susceptibility to skeletal disease. *Nat. Commun.* **12**, 2444 (2021).
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