Stem Cell Reports, Volume *11*

Supplemental Information

Pre-transplantational Control of the Post-transplantational Fate of Human Pluripotent Stem Cell-Derived Cartilage

John Y. Lee, Nadine Matthias, Azim Pothiawala, Bryan K. Ang, Minjung Lee, Jia Li, Deqiang Sun, Sebastien Pigeot, Ivan Martin, Johnny Huard, Yun Huang, and Naoki Nakayama

1. SUPPLEMENTAL FIGURES

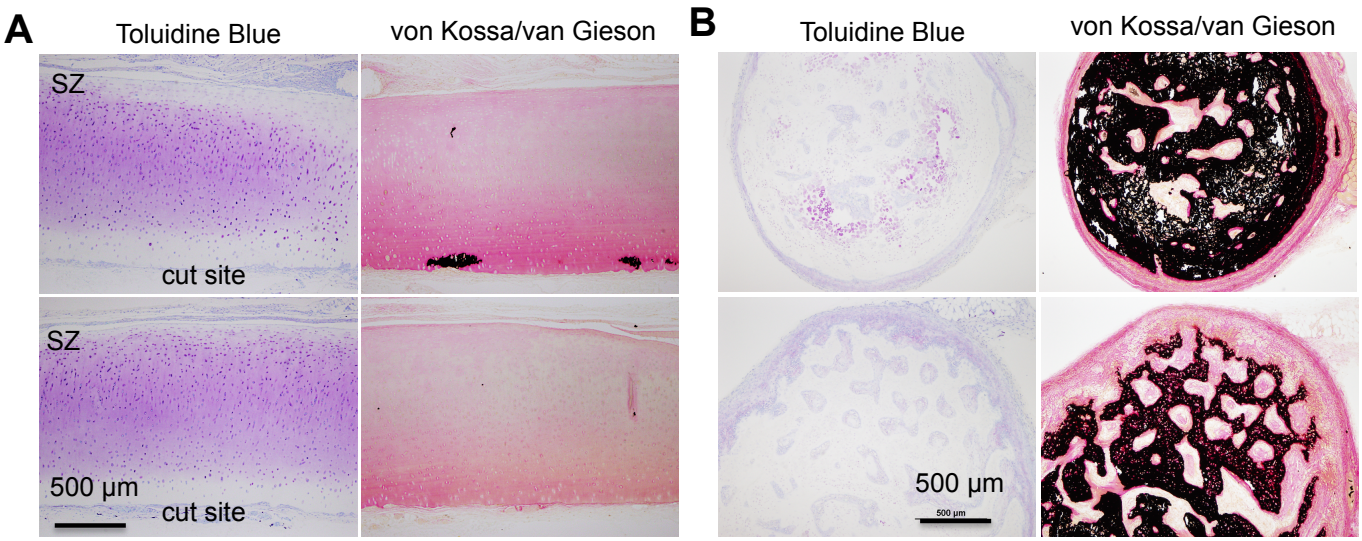


Fig. S1 *In vivo* cartilage stability assayed by subcutaneous transplantation into NSG mice. **(A) Bovine articular cartilage.** A piece of 2-year-old bovine knee articular cartilage subcutaneously transplanted for 12 weeks in NSG mice. Note that some areas of the cut site (in mid zone) but not the untouched natural surface (superficial zone, SZ) were mineralized. **(B) Cartilage pellet from FSbC-expanded human paraxial mesoderm.** Bony tissue with no cartilaginous areas (Toluidine Blue $[TB]^-$ von Kossa $[VK]^+$) detected 9 weeks after subcutaneous transplantation of cartilage pellets developed under PTB (PDGF+TGFβ+BMP4) conditions from expanded hESC-derived paraxial mesodermal cells (passage 4). They were more prone to mineralization than cartilage pellets made with uncultured paraxial mesoderm (Umeda, et al. 2015). Supplementary to Fig. 6.

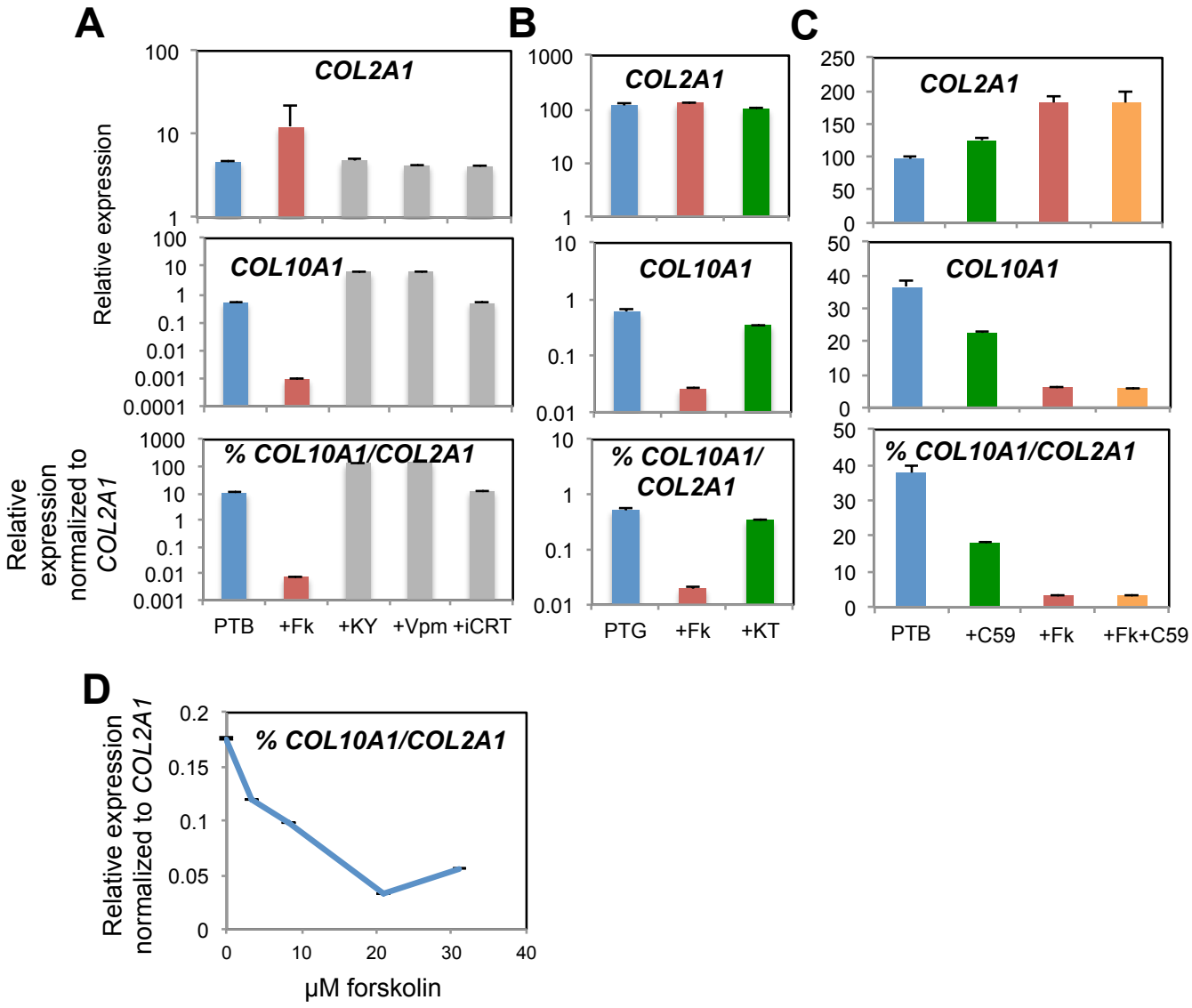


Fig. S2 Functional screening by RT-PCR of small molecules that suppress hypertrophic differentiation of chondrocytes.

(**A-C**) Chondrogenesis using hESC-derived ectomesenchymal cells was performed under PTB (PDGF+TGFβ+BMP4) or PTG (PDGF+TGFβ+GDF5) conditions with forskolin (Fk), KY02111 (KY), Verapamil (Vpm) or iCRT14 (iCRT) (**A**), with Fk or KT5823 (KT) (**B**), and with Fk, 30 nM Wnt-C59 (C59), or Fk+C59 (**C**). The small molecules were added on day 11, except for C59, which was added on day 24. Pellets were harvested on day 32. (**D**) Chondrogenesis using hESC-derived ectomesenchymal cells was performed with 3, 9, 20, and 30 μM forskolin. Supplementary to Fig. 1D-G.

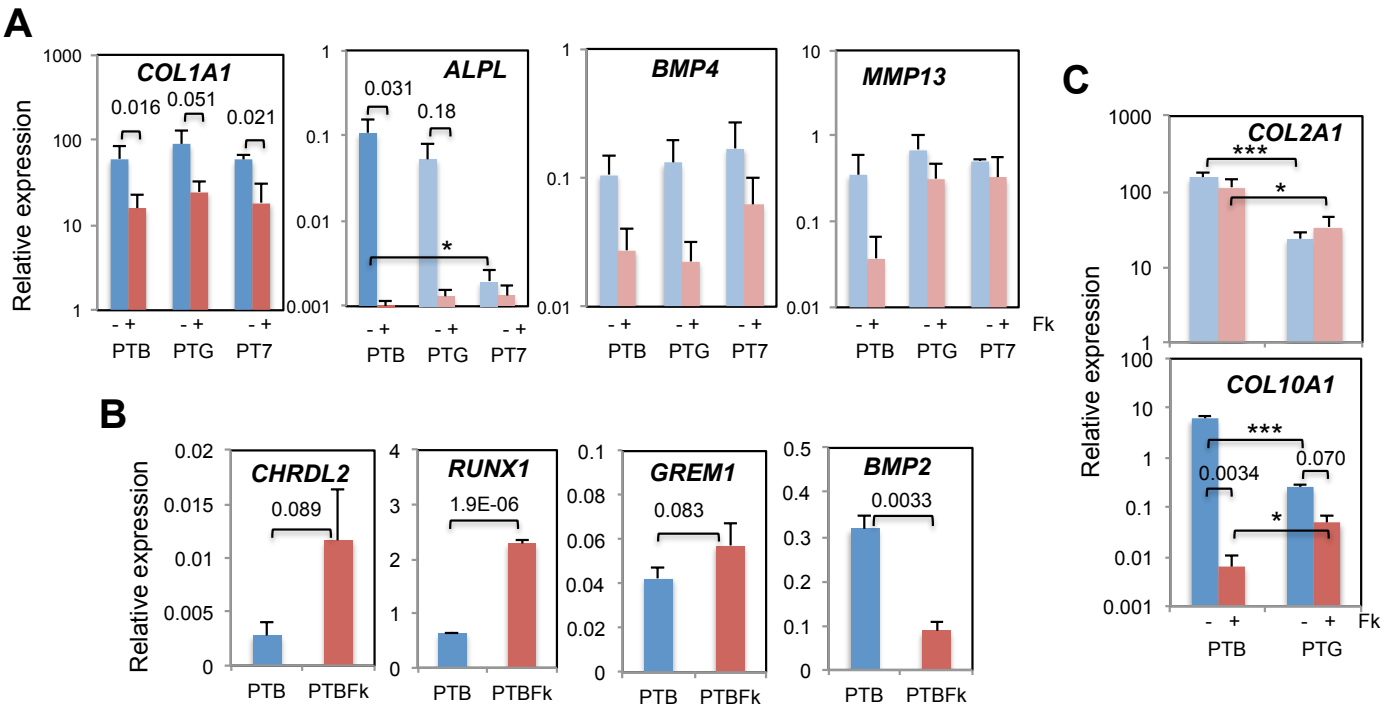


Fig. S3 Gene expression profiling by RT-PCR in forskolin-treated and untreated cartilage pellets. (**A, B**) Chondrogenesis using hESC-derived ectomesenchymal cells was performed under PTB (PDGF+TGFβ+BMP4), PTG (PDGF+TGFβ+GDF5), and PT7 (PDGF+TGFβ+BMP7) conditions, with (Brown: +Fk) or without (Blue: -Fk) forskolin. (**A**) Mean expression levels of chondrocyte genes are shown with SEM and *P*-values (+/-Fk comparison, values of *P*>0.2 are not shown). *COL1A1*: n (independent experiments)=7, *ALPL*: n=3-5, other comparisons **P*=0.10, *BMP4*: n=3, *MMP13*: n=3. Supplementary to Fig. 2A. (**B**) RT-PCR confirmation of RNA-seq data. n=3-4. Supplementary to Fig. 5EF. (**C**) Cartilage from SOX9-GFP hiPSC-derived ectomesenchymal cells made under PTB and PTG conditions with (brown) or without (blue) Fk. Mean expression levels with SEM and *P*-values (+/-Fk comparison) are shown (n=3-4). Supplementary to Fig. 1G. Other comparisons: *COL2A1*: **P*=0.070, ***P*=0.0035, *COL10A1*: **P*=0.083, ***P*=0.0034.

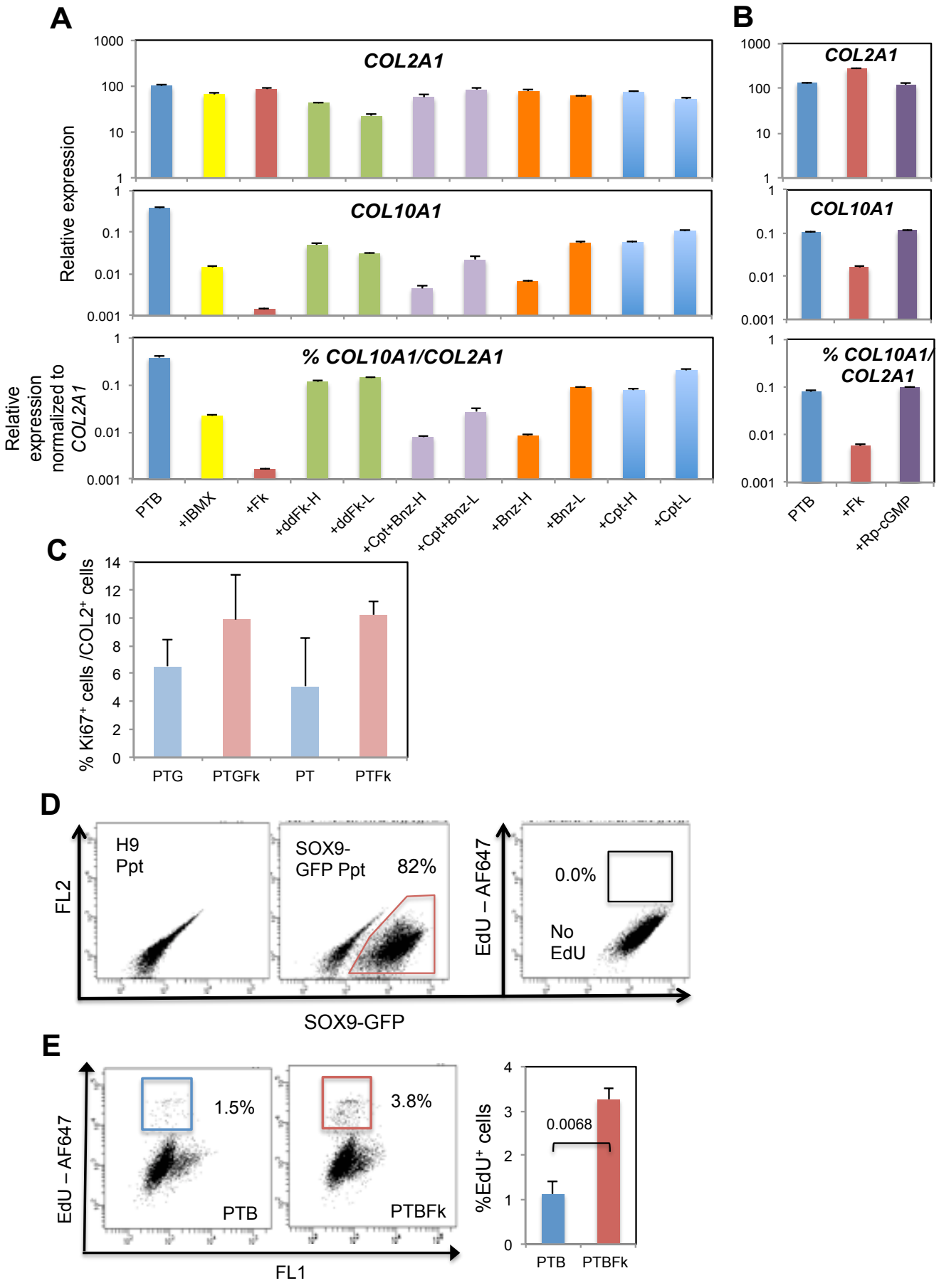

A
COL2A1
COL10A1
% COL10A1/COL2A1
Relative expression
Relative expression normalized to COL2A1
1000
100
10
1
0.1
0.01
0.001
PTB
+IBMX
+Fk
+ddFk-H
+ddFk-L
+Cpt+Bnz-H
+Cpt+Bnz-L
+Bnz-H
+Bnz-L
+Cpt-H
+Cpt-L
B
COL2A1
COL10A1
% COL10A1/ COL2A1
PTB
+Fk
+Rp-cGMP
C
% Ki67+ cells /COL2+ cells
14
12
10
8
6
4
2
0
PTG
PTGFk
PT
PTFk
D
H9 Ppt
SOX9-GFP Ppt
82%
0.0%
No EdU
FL2
EdU – AF647
SOX9-GFP
E
1.5%
3.8%
PTB
PTBFk
EdU – AF647
FL1
%EdU+ cells
0.0068
4
3
2
1
0
PTB
PTBFk

Fig. S4 Effect of cAMP/cGMP analogs on *COL10A1* expression during chondrogenesis.
Chondrogenesis using hESC-derived ectomesenchymal cells was performed under PTB (PDGF+TGFβ+BMP4) conditions for 33 days (**A**) with 0.5 mM IBMX, 25 μM forskolin (Fk), 12.5 μM (L) and 25 μM (H) 1,9-dideoxyforskolin (ddFk), 3 μM (L) and 6 μM (H) CPT-cAMP (Cpt), 40 μM (L) and 80 μM (H) Bnz-cAMP (Bnz), 3 μM CPT-cAMP+40 μM Bnz-cAMP (Cpt+Bnz-L), and 6 μM CPT-cAMP+80 μM Bnz-cAMP (Cpt+Bnz-H), and (**B**) with 25 μM forskolin (Fk), and 60 μM Rp-cGMP, added on day 11. Supplementary to Fig. 3A.

Forskolin effects on the proliferation of chondrocytes in cartilage pellets.
(**C**) Ki67 staining. PTG (PDGF+TGFβ+GDF5) vs. PTG+forskolin (PTGFk): *P*=0.40 (n=4). PT (PDGF+TGFβ) vs. PTFk: *P*=0.28 (n=2-3). Statistically insignificant differences. Supplementary to Fig. 3C (**D**) Without EdU labeling control for Fig. 3D. (**E**) EdU labeling of Fk treated and untreated cartilage pellets generated from H9 hESC-derived ectomesenchymal cells. Left Panels: representative FACS plots. Right panel: Mean % positive cells with SEM and *P*-value. n=3. Supplementary to Fig. 3D.

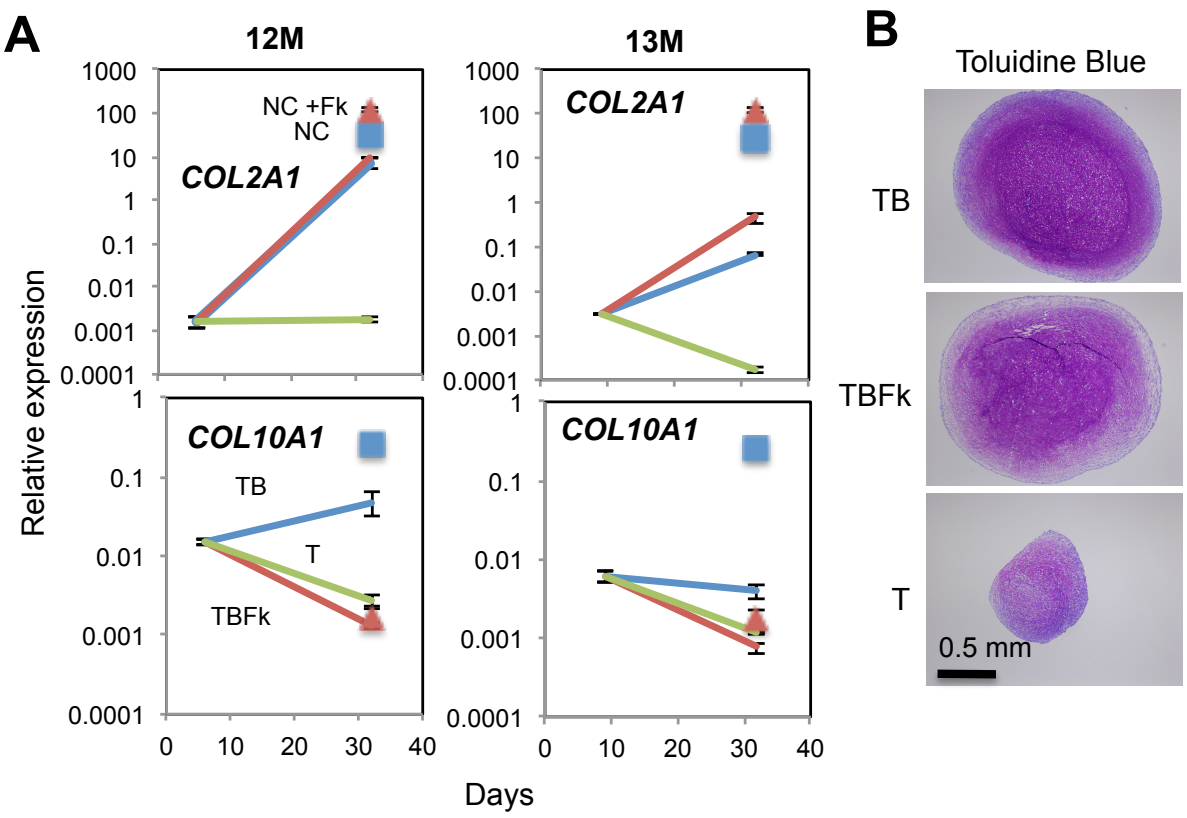


Fig. S5 Forskolin effects on chondrogenesis from dedifferentiated adult human nasal chondrocytes.

(**A**) Nasal chondrocytes from 2 male patients (12M, 13M) were individually differentiated in the presence of TGFβ alone (T) as described (Pelttari et al., 2014), or under modified conditions (TB: TGFβ+BMP4, and TBFk: TGFβ+BMP4+forskolin). On days 6 and 32, cartilage pellets were harvested and subjected to real-time RT-PCR. Supplementary to Fig. 4E. Blue square (NC): cartilage from hESC-ectomesenchymal cells formed under PTB (PDGF+TGFβ+BMP4), Brown triangle (NC+Fk): cartilage formed under PTBFk. (**B**) Supplementary to Fig. 4F. Toluidine blue (sGAG) staining of the same cartilage pellet samples.

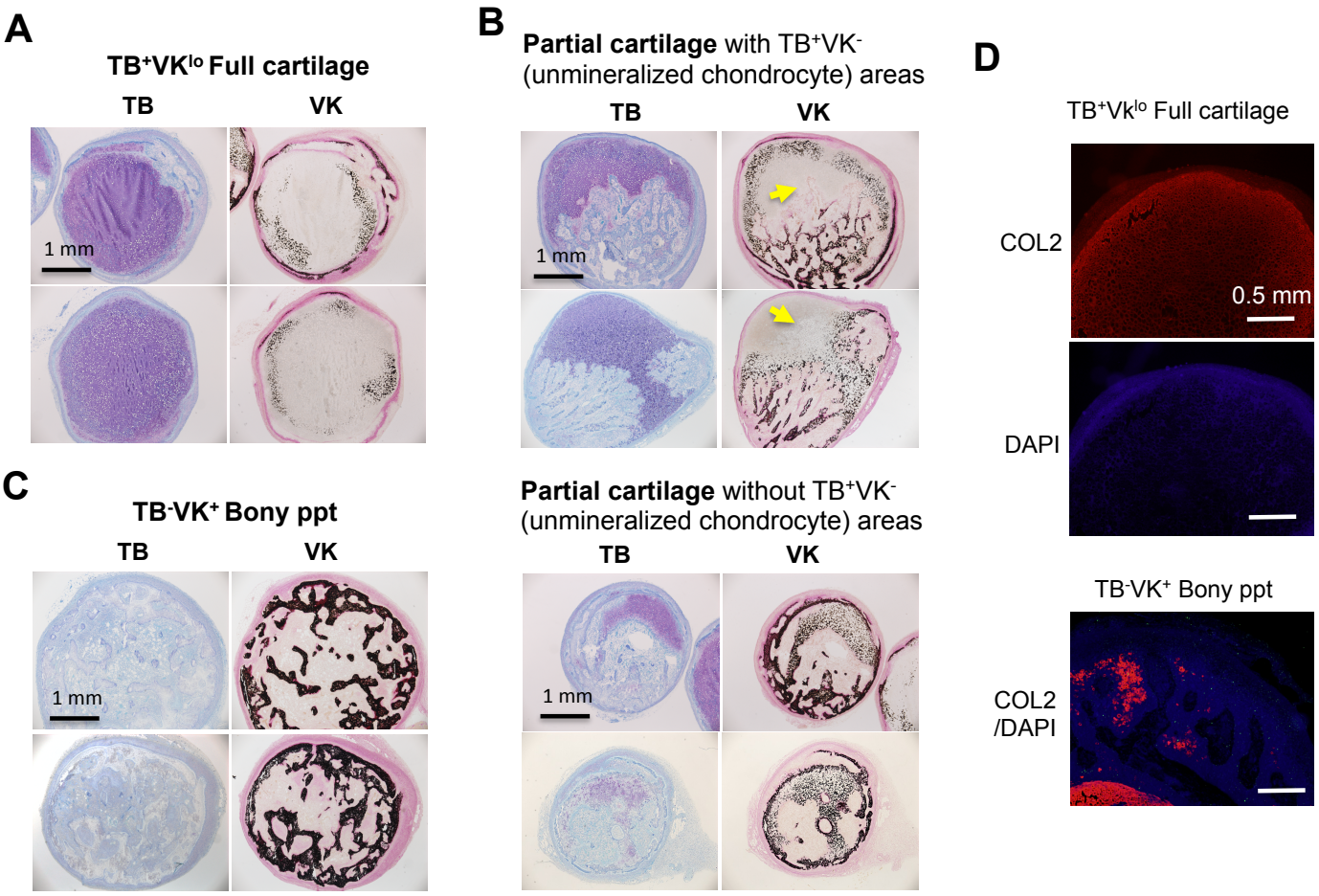
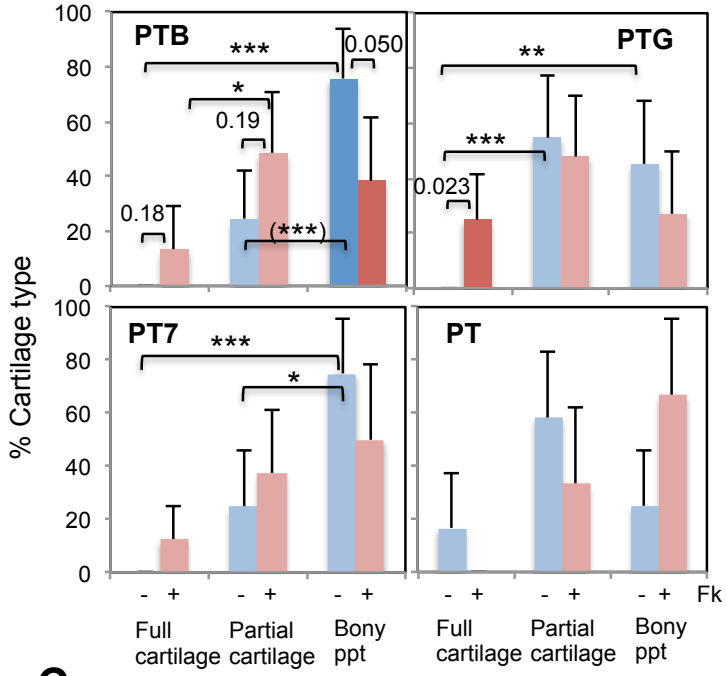
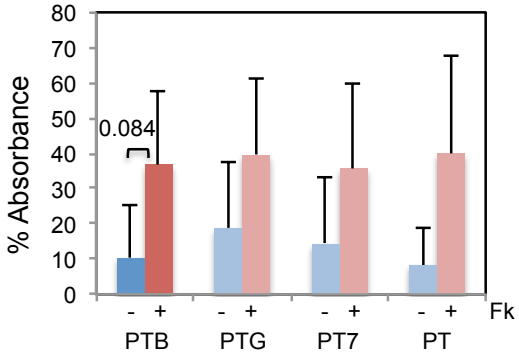


Fig. S6 Types of cartilage recovered from the 8-week ectopic transplantation.
(**A**) $TB^{+}VK^{lo}$ full cartilage: >80% area (usually the periphery of cartilage pellets did not stain) shows metachromatic (pink-purple) staining with Toluidine blue (TB) and consists largely of unmineralized chondrocytes. However, pellets often contained some mineralized chondrocytes that stained weakly with von Kossa (VK). (**B**) Partial cartilage containing bony area and cartilaginous area. Upper panel: The cartilaginous area contains groups of unmineralized chondrocytes (yellow arrows). Lower panels: The cartilaginous area contains mostly mineralized chondrocytes. (**C**) $TB^{-}VK^{+}$ bony pellet (ppt): Mostly consisting of bony areas stained densely with VK, often containing $TB^{-}VK^{-}$ marrow-like areas, but without TB^{+} cartilaginous area. (**A-C**) Supplementary to Fig. 6B. (**D**) Lower magnification (using x4 objective lens) photos of the immunofluorescence staining of the Fig. 6C post-transplantation pellets.

A



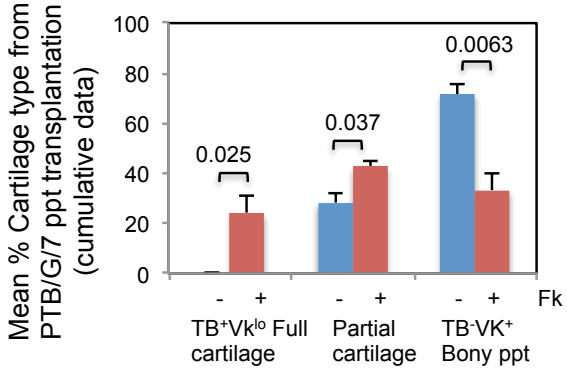
B



C

Ppt culture condition	$TB^{+}VK^{lo}$ Full cartilage (% viable ppts)	Partial cartilage (% viable ppts)	$TB^{-}VK^{+}$ Bony ppt (% viable ppts)	Recovered (viable) ppts	Absorbed ppts (% total ppts)	Transplanted (total) ppts
PTB	0 (0)	5 (24)	16 (76)	21	3 (13)	24
PTBFk	2 (12)	8 (47)	7 (41)	17	8 (32)	25
PTG	0 (0)	6 (36)	7 (64)	13	3 (19)	16
PTGFk	5 (36)	6 (43)	5 (21)	16	7 (30)	23
PT7	0 (0)	2 (25)	6 (75)	8	3 (27)	11
PT7Fk	2 (25)	3 (38)	3 (38)	8	3 (27)	11
PT	1 (14)	5 (57)	2 (29)	8	1 (11)	9
PTFk	0 (0)	1 (33)	2 (67)	3	4 (57)	7

D



E

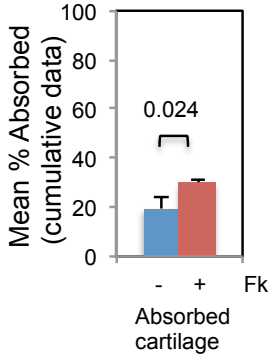


Fig. S7 Post-transplantation cartilage phenotypes:
Statistical analyses.
(**A**) Recovery of each of $TB^{+}VK^{lo}$ full cartilage, partial cartilage, and $TB^{-}VK^{+}$ bony pellet (ppt) after the 8-week ectopic transplantation of forskolin (Fk)-treated (Brown) and untreated (Blue) cartilage pellets was averaged and plotted with corresponding SEM and *P*-value (+/-Fk comparison, values of *P*>0.2 are not shown). PTB (PDGF+TGFβ+BMP4): n=9-10. PTG (PDGF+TGFβ+GDF5): n=7-8, PT7 (PDGF+TGFβ+BMP7): n=5-6, PT (PDGF+TGFβ): n=3-6. Other comparisons: PTB: **P*=0.060, ****P*=9.1E-07, $^{(***)}$*P*=0.0032, PTG: ***P*=0.023, ****P*=0.0082, PT7: **P*=0.065, ****P*=0.0014. Supplementary to Fig. 6D. (**B**) Pellet loss after the 8-week ectopic transplantation from each transplantation experiment was averaged and plotted with corresponding SEM and *P*-values (+/-Fk comparison). Supplementary to Fig. 6D.
Cumulative data analyses.
(**C**) The number of recovered $TB^{+}VK^{lo}$ full cartilage, partial cartilage, and $TB^{-}VK^{+}$ bony pellets (ppts) as well as the number of absorbed pellets after the 8-week ectopic transplantation of forskolin (Fk)-treated (Brown) and untreated (Blue) cartilage pellets were summed. Supplementary to Fig. 6D. (**D**) The % Full cartilage, % Partial cartilage and % Bony pellet (ppt) values from transplanted pellets produced under PTB (PDGF+TGFβ+BMP4), PTG (PDGF+TGFβ+GDF5) and PT7 (PDGF+TGFβ+BMP7) conditions, and those from PTB+Fk (PTBFk), PTGFk and PT7Fk conditions, shown in (**C**) were averaged and plotted with SEM with *P*-values. Supplementary to Fig. 6E **(E**) The % absorbed pellet values from transplanted pellets produced under PTB, PTG and PT7 conditions and those from PTBFk, PTGFk and PT7Fk conditions, shown in (**C**) were also averaged and plotted with SEM and *P*-value. Supplementary to Fig. 6E.

2. SUPPLEMENTAL TABLES

Table S1. Top GO Terms (Biological Process) *P*<1.0E-10

Top 20	Gene set names	P	FDR q
Fk induced genes	**（PTBFk＞PTB）**		
GO:0007059	chromosome segregation	5.71E-17	8.86E-13
GO:0098813	nuclear chromosome segregation	5.86E-17	4.54E-13
GO:0000819	sister chromatid segregation	1.57E-16	8.11E-13
GO:0007275	multicellular organism development	1.89E-16	7.31E-13
GO:0048856	anatomical structure development	3.10E-16	9.61E-13
GO:0032502	developmental process	6.38E-16	1.65E-12
GO:0048731	system development	1.06E-15	2.34E-12
GO:0032501	multicellular organismal process	3.09E-15	5.98E-12
GO:0000280	nuclear division	8.68E-15	1.49E-11
GO:0000278	mitotic cell cycle	1.25E-14	1.94E-11
GO:0051301	cell division	1.26E-14	1.77E-11
GO:0048285	organelle fission	3.94E-14	5.09E-11
GO:0022402	cell cycle process	7.15E-14	8.53E-11
GO:1903047	mitotic cell cycle process	1.26E-13	1.40E-10
GO:0007049	cell cycle	2.13E-13	2.20E-10
GO:0000070	mitotic sister chromatid segregation	7.61E-13	7.37E-10
GO:0140014	mitotic nuclear division	7.86E-13	7.17E-10
GO:0051239	regulation of multicellular organismal process	6.59E-12	5.67E-09
GO:0009790	embryo development	1.16E-11	9.48E-09
GO:0009653	anatomical structure morphogenesis	1.85E-11	1.44E-08
Fk suppressed genes	**（PTB＞PTBFk）**		
GO:0048731	system development	1.66E-17	2.57E-13
GO:0007275	multicellular organism development	2.57E-17	1.99E-13
GO:0048856	anatomical structure development	3.92E-16	2.02E-12
GO:0009888	tissue development	1.76E-15	6.83E-12
GO:0032502	developmental process	3.88E-15	1.2E-11
GO:0032879	regulation of localization	1.04E-12	2.7E-09
GO:0032501	multicellular organismal process	1.36E-12	2.64E-09
GO:0048513	animal organ development	2.26E-12	3.9E-09
GO:0007154	cell communication	3.71E-12	5.75E-09
GO:0050793	regulation of developmental process	6.68E-12	7.97E-09
GO:0051239	regulation of multicellular organismal process	7.03E-12	7.78E-09
GO:0009653	anatomical structure morphogenesis	1.1E-11	1.0E-08

GO:0023052	signaling	4.25E-11	3.66E-08

Table S2. Tissue/cell development-related GO Terms P<1.0E-04

Developmental GO	Gene set names	P	FDR q
Fk induced genes	**（PTBFk＞PTB）**		
GO:0007399	nervous system development	4.12E-10	2.56E-07
GO:0035295	tube development	2.46E-07	5.69E-05
GO:1901342	regulation of vasculature development	3.42E-07	7.70E-05
GO:0048514	blood vessel morphogenesis	3.62E-07	7.90E-05
GO:0001525	angiogenesis	5.80E-07	1.15E-04
GO:0045765	regulation of angiogenesis	8.33E-07	1.17E-04
GO:0022414	reproductive process	2.14E-06	3.29E-04
GO:0000003	reproduction	2.17E-06	3.31E-04
GO:0001568	blood vessel development	2.85E-06	4.06E-04
GO:0001655	urogenital system development	2.98E-06	4.12E-04
GO:0051960	regulation of nervous system development	4.03E-06	5.26E-04
GO:0001944	vasculature development	6.68E-06	8.04E-04
GO:0022008	neurogenesis	6.72E-06	8.02E-04
GO:0001501	skeletal system development	7.19E-06	8.45E-04
GO:0048699	generation of neurons	1.05E-05	1.16E-03
GO:0072358	cardiovascular system development	1.29E-05	1.39E-03
GO:0048608	reproductive structure development	1.73E-05	1.81E-03
GO:0061458	reproductive system development	1.98E-05	2.02E-03
GO:2000181	negative regulation of blood vessel morphogenesis	2.72E-05	2.58E-03
GO:0072001	renal system development	3.41E-05	3.10E-03
GO:0001822	kidney development	3.81E-05	3.36E-03
GO:1901343	negative regulation of vasculature development	4.86E-05	4.12E-03
GO:0061448	connective tissue development	5.54E-05	4.64E-03
GO:0001657	ureteric bud development	8.18E-05	6.58E-03
GO:0051216	cartilage development	8.56E-05	6.64E-03
GO:0060429	epithelium development	8.84E-05	6.72E-03
GO:0072164	mesonephric tubule development	9.00E-05	6.78E-03
GO:0072163	mesonephric epithelium development	9.00E-05	6.74E-03
GO:0046546	development of primary male sexual characteristics	9.10E-05	6.78E-03
Fk suppressed genes	**（PTB＞PTBFk）**		
GO:0048699	generation of neurons	2.54E-09	1.31E-06
GO:0022008	neurogenesis	1.51E-08	6.31E-06
GO:0030182	neuron differentiation	3.11E-08	1.18E-05
GO:0001503	ossification	7.79E-08	2.42E-05

GO:0060537	muscle tissue development	1.45E-07	4.24E-05
GO:0007399	nervous system development	2.01E-07	5.37E-05
GO:0014706	striated muscle tissue development	2.16E-07	5.68E-05
GO:0060429	epithelium development	3.02E-07	7.31E-05
GO:0072001	renal system development	3.90E-07	8.77E-05
GO:0007517	muscle organ development	4.02E-07	8.78E-05
GO:0072359	circulatory system development	5.47E-07	1.10E-04
GO:0001501	skeletal system development	6.34E-07	1.24E-04
GO:0001822	kidney development	1.44E-06	2.56E-04
GO:0030278	regulation of ossification	1.76E-06	2.90E-04
GO:0035239	tube morphogenesis	1.98E-06	3.21E-04
GO:0060562	epithelial tube morphogenesis	2.41E-06	3.81E-04
GO:0001655	urogenital system development	2.84E-06	4.27E-04
GO:0045664	regulation of neuron differentiation	4.50E-06	6.34E-04
GO:0016202	regulation of striated muscle tissue development	4.65E-06	6.44E-04
GO:0061061	muscle structure development	4.99E-06	6.84E-04
GO:1901861	regulation of muscle tissue development	5.54E-06	7.47E-04
GO:0035295	tube development	6.06E-06	7.90E-04
GO:0048514	blood vessel morphogenesis	6.14E-06	7.86E-04
GO:0048634	regulation of muscle organ development	6.59E-06	8.30E-04
GO:0051960	regulation of nervous system development	9.35E-06	1.08E-03
GO:0003007	heart morphogenesis	1.07E-05	1.21E-03
GO:0045165	cell fate commitment	1.20E-05	1.35E-03
GO:0001649	osteoblast differentiation	1.40E-05	1.51E-03
GO:0031214	biomineral tissue development	1.81E-05	1.90E-03
GO:0007507	heart development	1.90E-05	1.97E-03
GO:0050767	regulation of neurogenesis	2.24E-05	2.23E-03
GO:0001568	blood vessel development	2.66E-05	2.58E-03
GO:0048641	regulation of skeletal muscle tissue development	3.00E-05	2.87E-03
GO:0048666	neuron development	3.48E-05	3.20E-03
GO:0060348	bone development	3.93E-05	3.48E-03
GO:2000725	regulation of cardiac muscle cell differentiation	4.48E-05	3.88E-03
GO:0001525	angiogenesis	4.48E-05	3.86E-03
GO:0055024	regulation of cardiac muscle tissue development	5.34E-05	4.38E-03
GO:0048738	cardiac muscle tissue development	5.84E-05	4.74E-03
GO:0001944	vasculature development	7.16E-05	5.64E-03
GO:2000726	negative regulation of cardiac muscle cell differentiation	8.15E-05	6.28E-03
GO:0030509	BMP signaling pathway	8.82E-05	6.71E-03

Table S3. Fk-induced gene list.

Skeletogenesis-related genes	PTB DESeq	PTBFk DESeq	fold induction by Fk ≥2.0	FDR adjusted P<0.05
DLK1	15532.21	50776.97	3.27	7.04E-09
MMP2	7223.26	24243.16	3.36	7.85E-08
CILP2	9466.24	19658.75	2.08	4.22E-05
SFRP5	1947.04	6689.51	3.44	4.86E-02
SFRP1	1206.22	4896.12	4.06	1.84E-07
PBX3	2120.04	5234.18	2.47	2.55E-16
RUNX1	1278.92	3929.79	3.07	7.03E-16
PTN	697.46	2631.56	3.77	5.87E-08
TNFRSF11B	1198.53	2595.39	2.17	7.90E-03
SOX11	1244.26	2500.55	2.01	6.39E-07
SIX2	857.18	2380.29	2.78	2.34E-10
CITED2	879.18	2051.24	2.33	3.60E-09
GATA3	887.45	1870.27	2.11	2.18E-03
ALX4	648.05	1803.80	2.78	2.77E-10
EYA1	517.90	1625.08	3.14	9.49E-15
FAP	407.94	1371.01	3.36	4.16E-02
INHBA	370.15	1321.50	3.57	4.34E-07
ITGA4	765.55	2255.70	2.95	5.45E-11
GATA2	254.42	747.22	2.94	1.06E-03
SOCS3	317.62	695.17	2.19	1.87E-02
RSPO3	50.16	597.70	11.94	9.85E-23
TGFBR3	56.25	377.66	6.73	1.03E-10
RSPO2	59.30	202.07	3.42	1.24E-02
LHX8	74.28	152.34	2.04	1.84E-02
CSF3R	6.60	77.04	11.63	8.39E-05
UCMA	1.60	68.10	43.15	1.03E-06
LHX6	15.71	45.90	2.90	4.81E-02
DACT2	1.83	21.03	11.19	1.65E-03
RARRES1	3.65	15.40	4.20	2.71E-02
DLL4	0.29	8.27	24.42	2.23E-02
BMP signaling genes	**PTB** DESeq	**PTBFk** DESeq	**fold induction by Fk ≥2.0**	**FDR adjusted P<0.05**
GREM1	1346.97	4501.42	3.34	4.40E-08
GDF5	69.95	159.11	2.28	5.66E-04
BMP5	38.96	120.17	3.09	2.66E-02
CHRDL2	11.44	83.24	7.23	3.41E-09
BMPER	5.65	32.00	5.82	2.57E-03

Cell cycle control genes	PTB DESeq	PTBFk DESeq	fold induction by Fk ≥2.0	FDR adjusted P<0.05
CCNB1	336.49	690.73	2.06	1.64E-02
CCNA2	236.81	592.52	2.51	1.00E-02
CDK1	166.77	584.12	3.51	2.22E-02
CDK18	144.72	465.27	3.21	2.62E-04
CCNB2	113.80	287.64	2.54	7.50E-04
E2F1	101.41	232.61	2.30	1.41E-03
CCNE2	47.18	105.64	2.23	3.27E-03
MYCN	26.06	95.27	3.63	8.36E-03
CDC45	22.95	55.67	2.45	9.40E-03
CDC25C	14.73	47.28	3.24	2.20E-02
E2F8	11.04	41.65	3.82	6.59E-03
CCNA1	4.44	19.91	4.50	8.17E-03
DNA replication genes	**PTB** DESeq	**PTBFk** DESeq	**fold induction by Fk ≥2.0**	**FDR adjusted P<0.05**
ORC6	94.27	190.00	2.02	2.50E-02
MCM10	43.79	125.85	2.88	1.17E-02
CDT1	46.94	109.89	2.35	5.77E-03
ORC1	30.51	75.11	2.47	5.11E-03

Table S4. Fk-suppressed gene list.

Skeletogenesis-related genes	PTB DESeq	PTBFk DESeq	fold suppression by Fk ≥2.0	FDR adjusted P<0.05
COL10A1	759335.21	27191.44	27.93	1.42E-11
PTH1R	57556.05	20996.49	2.74	1.25E-07
CYTL1	20403.47	6378.10	3.20	4.21E-02
MEF2C	19516.42	8279.90	2.36	8.25E-05
IHH	16969.11	3656.91	4.64	1.10E-03
IBSP	16004.16	85.41	187.55	4.17E-09
IGF1R	13863.53	5317.81	2.61	1.57E-33
NRCAM	7953.61	1665.61	4.78	1.15E-03
GSK3B	6291.89	2568.07	2.45	1.92E-17
RUNX2	5022.87	1617.64	3.11	2.93E-06
ALPL	4578.90	152.96	29.96	5.13E-09
FST	4174.02	953.38	4.38	3.21E-03
SP7	3828.53	646.53	5.92	3.31E-10
FOXA2	3504.24	994.51	3.52	1.53E-11
IRX5	2042.96	310.43	6.59	1.03E-10
CD24	2006.71	213.78	9.40	3.49E-05
WNT5B	1648.18	610.88	2.70	3.62E-07
DKK1	1516.17	64.48	23.42	1.04E-07
WNT11	1479.72	363.90	4.06	5.93E-08
VDR	1478.11	428.00	3.45	6.62E-12
VAV3	1406.94	97.05	14.52	8.08E-07
DLX5	1256.06	593.71	2.12	1.29E-02
WNT16	1914.44	290.74	6.58	7.95E-03
IL17B	1070.22	43.11	24.66	4.55E-35
JAG2	896.56	430.89	2.08	1.49E-08
COL4A6	759.47	116.31	6.55	2.48E-16
SPP1	755.78	21.22	35.38	6.60E-06
DACH1	529.83	41.79	12.84	9.47E-15
AXIN2	328.09	154.90	2.12	1.15E-03
DLL1	310.31	45.14	6.94	2.20E-09
KL	191.59	62.53	3.05	1.18E-05
MEPE	187.06	1.02	186.09	9.20E-16
CUX2	175.05	0.79	232.46	2.60E-16
ANK1	141.71	8.88	16.55	7.71E-12
WNT10B	138.31	22.82	6.05	2.43E-09
DLX3	93.87	26.88	3.55	1.15E-04
DKK2	63.92	7.89	7.96	1.58E-02
DMP1	46.19	9.41	4.77	2.58E-02

MYOCD	28.52	3.55	8.39	9.44E-04
CYSLTR1	24.29	0.00	127.12	4.72E-06
DCX	16.86	3.81	4.30	1.86E-02
CALCRL	13.48	0.75	17.85	2.52E-03
BMP signaling genes	**PTB** DESeq	**PTBFk** DESeq	**fold suppression by Fk ≥2.0**	**FDR adjusted P<0.05**
DCN	27128.19	13080.70	2.07	2.15E-02
SMPD3	5685.56	308.89	18.41	1.22E-06
ID2	2699.09	1246.36	2.16	5.73E-07
BMP2	2153.56	540.41	3.99	6.72E-11
CHRDL1	411.70	189.02	2.18	9.14E-11
BMP8A	254.56	105.41	2.43	5.90E-04
BMP8B	34.98	10.06	3.51	1.98E-02
Cell cycle control genes	**PTB** DESeq	**PTBFk** DESeq	**fold suppression by Fk ≥2.0**	**FDR adjusted P<0.05**
CDKN1A	8873.45	3582.71	2.48	1.50E-16
CDC42EP3	3631.13	1647.02	2.21	1.93E-05
MYCL	34.37	11.45	2.95	1.15E-02
DNA replication genes	**PTB** DESeq	**PTBFk** DESeq	**fold suppression by Fk ≥2.0**	**FDR adjusted P<0.05**

Table S5. Comparative bioinformatics analysis between Fk-induced genes with Wu, et al.'s human embryonic chondrocytes.

Fk-induced (fold induction ≥2.0) genes	17w articular chondrocytes Microarray unit	6w condensing chondrocytes Microarray unit	17w > 6w (fold increase >1.75)
ST6GALNAC3	9.51	4.05	2.34
LIX1	18.04	9.49	1.90
MXRA5	11.42	6.12	1.87
CENPK	8.70	4.70	1.85
KIT	7.51	4.09	1.84
JAKMIP2	13.90	7.66	1.81
BAI3	12.07	6.87	1.76

Fk-induced (fold induction ≥2.0) genes	17w articular chondrocytes Microarray unit	6w condensing chondrocytes Microarray unit	17w < 6w (fold increase >1.75)
CHI3L2	4.97	11.54	2.32
ICAM1	16.76	38.67	2.31
SERPINA3	5.28	12.02	2.28
ITIH6	4.25	9.19	2.16
BHLHE40	10.85	23.16	2.14
C1S	9.30	19.58	2.11
NNMT	4.67	9.74	2.08
GPRC5A	15.35	31.18	2.03
TRAF1	9.15	18.47	2.02
TNFRSF11B	11.07	21.75	1.96
PLA2G2A	6.35	11.88	1.87
ATP6V0A4	5.74	10.58	1.84
OSMR	17.59	31.94	1.82
INSC	4.31	7.61	1.77
CST1	4.07	7.14	1.76

Table S6. Comparative bioinformatics analysis between Fk-suppressed genes with Wu, et al.'s human embryonic chondrocytes.

Fk-suppressed (fold reduction ≥2.0) genes	17w articular chondrocytes Microarray unit	6w condensing chondrocytes Microarray unit	17w > 6w (fold increase >1.75)
DKK1	8.76	3.55	2.47
DACH1	23.78	10.05	2.37
CDH10	7.35	3.24	2.27
DCX	18.47	9.52	1.94
FGF13	10.04	5.67	1.77
GPR64	9.31	5.32	1.75

DKK1 and *DCX* are articular cartilage genes.
Forskolin suppresses the expression of these articular cartilage genes.

Fk-suppressed (fold reduction ≥2.0) genes	17w articular chondrocytes Microarray unit	6w condensing chondrocytes Microarray unit	17w < 6w (fold increase >1.75)
AGT	3.15	13.09	4.15
ISG20	9.61	25.49	2.65
AOC3	3.57	7.21	2.02
SLC28A3	7.26	14.12	1.95
PPP4R4	11.89	21.47	1.81
LOC100126784	13.15	23.57	1.79

Table S7. n (independent experiment number) and *P*-value lists.

Gene	*P*, n	*P* and n values for each condition		
Fig. 2B		**PTBFk/PTB**	**PTGFk/PTG**	**PT7Fk/PT7**
COL2A1	*P*	0.10	0.28	0.17
	n	10	8	6
COL10A1	*P*	5.4E-21	1.1E-08	4.1E-10
	n	17	11	9
COL1A1	*P*	2.5E-03	1.7E-04	6.3E-03
	n	7	7	6
ALPL	*P*	8.6E-04	0.016	0.57
	n	6	3	3
SOX9	*P*	0.32	0.26	0.20
	n	9	3	3
RUNX2	*P*	1.9E-06	0.0097	0.14
	n	10	3	3
BMP4	*P*	0.0081	0.0017	1.0E-04
	n	3	3	3
MMP13	*P*	0.12	0.68	0.63
	n	3	3	3
Fig. 3B Left		**+cAMP/-cAMP**	**+Fk/-Fk**	**+IBMX/-IBMX**
COL2A1	*P*	0.35	0.037	0.30
	n	8	10	8
COL10A1	*P*	9.3E-06	7.9E-09	4.2E-05
	n	6	8	8
Fig. 3B Right		**+Fk/-Fk**	**+C59/-C59**	**+Fk+C59/-Fk-C59**
COL2A1	*P*	0.28	0.36	0.35
	n	5	6	6

COL10A1	*P*	5.9E-06	0.0077	1.8E-06
	n	5	6	6
	P	0.0031		

3. SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Human pluripotent stem cell culture.

H9 hESCs were maintained on mouse embryonic fibroblast feeder cells in SR medium: Dulbecco's modified Eagle's medium (DMEM): Ham's F12 (1:1), 20% (v/v) KnockOut Serum Replacement (KSR), 2 mM GlutaMAX, 0.1 mM non-essential amino acids (all from Invitrogen, Carlsbad, CA) and 90 μM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), supplemented with 4 ng/ml FGF2 (hFGF2-IS, Miltenyi Biotec, Bergisch Gladbach, Germany) as described (Umeda et al., 2012), Alternatively CY2-SOX9-2A-ZsGreen-2A-Puro (SOX9-GFP) hiPSCs derived from the former Center for Regenerative Medicine in NIH were maintained in Essential-8 medium on a vitronectin-coated plate (Invitrogen). Media and supplements, buffers and specialized tissue culture plates were generally sourced as described (Umeda et al., 2015; Umeda et al., 2012; Wang and Nakayama, 2009; Wang et al., 2010).

Generation and expansion of ectomesenchymal cells from hPSCs through neural crest specification.

Human PSCs were treated with collagenase I (Worthington, Lakewood, NJ) to generate clumps, which were plated onto a low-adhesion dish (Sarstedt, Nümbrecht, Germany) and cultured for 1 day in a chemically defined medium (CDM): Iscove's modified Dulbecco's Medium (IMDM) (Sigma): Ham's F12 (Invitrogen) (1:1), 0.5% (w/v) bovine serum albumin (Sigma), 1% (v/v) synthetic lipid concentrate (Invitrogen), 100 μg/ml human holo-transferrin (Sigma), 20 μg/ml human insulin (Sigma), 0.17 mM ascorbic acid-2-phosphate (AA2P, Sigma), 2 mM GlutaMAX (Invitrogen), and 0.4 mM monothioglycerol (MTG, Sigma), supplemented with 10 μM SB431542 (Tocris) and 5 μM Y27632 (ROCK inhibitor, Tocris, Ellisville MO) at 37^{o}C under 5% CO_2/5% O_2. The embryoid bodies (EBs) formed were transferred to a plate coated with 0.1% (w/v) porcine gelatin (Sigma) on day 1 and cultured in CDM with 10 μM SB431542 and 2 μM CHIR99021 (Tocris) till day 5. Then media was changed to CDM with 10 μM SB431542 only. On the day of

harvest (usually day 6), a suspension of single cells was prepared using TrypLE Select (Invitrogen), diluted 2.5-fold with 0.5 mM EDTA in D-PBS (without Ca^{2+} and Mg^{2+}), immunostained with the AlexaFluor647-conjugated mouse anti-hCD271 (#560326, BD Biosciences, San Jose, CA) and phycoerythrin (PE)-conjugated mouse anti-hCD73 (#344004, BioLegend, San Diego, CA) monoclonal antibodies, and the $CD271^{hi}CD73^{-}$ cell population was isolated by FACS as described (Umeda et al., 2015). Alternatively, the $CD271^{hi}$ cell population was isolated by magnetic cell sorting using the MACS cell separation system and CD271 microbead kits (#130-092-283, Miltenyi Biotec). The sorted cells were transferred to a plate coated with 10 μg/ml human fibronectin (Sigma) and cultured overnight at 4°C in CDM supplemented with 2 μg/ml heparin (Sigma), 2.5 μg/ml catalase (Sigma), 5-10 ng/ml FGF2 and 5-7.5 μM SB431542 (FSb medium), at 37°C under 5% CO_2/5% O_2. The cells were passaged every 2-3 days at 3-4 x 10^4 cells/cm^2 using the diluted TrypLE Select as described (Umeda et al., 2015). At the desired density, the ectomesenchymal cells were primed with CDM supplemented with 5 ng/ml FGF2 and 10 ng/ml TGFβ3 (hTGFβ3, R&D Systems, Minneapolis, MN) for 3 days prior to chondrogenesis induction, and harvested with TrypLE Select.

Generation and isolation of paraxial mesoderm from hPSCs.

Human PSCs were differentiated 3-dimensionally using the CDM-based EB-forming culture as described (Umeda et al., 2015; Umeda et al., 2012) with slight modifications. Briefly, the EB culture was initiated in the CDM with extra anti-oxidants: 2.5 μg/ml catalase, 1.5 μg/ml reduced glutathione (Sigma) and 5 mM proline (Sigma) (CDM-AO medium), supplemented with 5 μM Y27632, 5 ng/ml BMP4 (hBMP4, R&D), and 5 μM CHIR99021, and maintained at 37°C under 5% CO_2/5% O_2. On day 1, medium was changed to CDM-AO containing 5 μM CHIR99021. On day 2, 100-500 ng/ml Noggin (mNoggin-Fc, R&D) was added. On day 3, EBs were transferred to CDM-AO containing 0.9% (w/v) methylcellulose (Methocel A4M, Dow Chemical, Midland, MI) with 100 ng/ml Noggin, 0.5-2 μM CHIR99021, 0.2 μM PD173074 (FGFR1 inhibitor, Tocris), 3

μM SB431542 and 5 ng/ml PDGF (hPDGF-BB, R&D). On day 6 or 7, single EB cells were obtained by treatment of EBs with the diluted TrypLE Select for 3-5 min at 37°C. The cells were stained with mouse anti-hPDGFRα (IgG_{2a}, #556001, BD) and anti-KDR (IgG_1, #101-M20 ReliaTech, Wolfenbüttel, Germany) monoclonal antibodies, then with biotin-conjugated goat anti-mouse IgG_{2a} (#1080-08), and PE-conjugated goat anti-mouse IgG_1 antibodies (#1070-01) from SouthernBiotech (Birmingham, AL), and finally with allophycocyanin (APC)-conjugated streptavidin (#554067, BD). The $KDR^-PDGFR\alpha^+$ cell population was isolated by FACS as described (Umeda et al., 2012).

Expansion of hPSC-derived paraxial mesoderm cells.

The FACS-isolated cells were cultured on a fibronectin coated plate in CDM supplemented with 2 μg/ml heparin, 2.5 μg/ml catalase, 5-10 ng/ml FGF2, 5-10 ng/ml PDGF, 5-7.5 μM SB431542 and 2 μM CHIR99021 (FPSbC medium), at 37°C under 5% CO_2/5% O_2 and passaged every 2-3 days at 3.0 x 10^4 cells/cm^2 using the diluted TrypLE Select. At passage 2, the medium composition was changed to FSbC by removal of PDGF from the FPSbC medium.

Expansion of human adult nasal chondrocytes.

Human adult nasal chondrocytes from one female (11F) and two male patients (12M, 13M) were independently isolated as described (Centola et al., 2013). Expansion of the chondrocytes was performed in DMEM (high glucose), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 25 mM HEPES buffer, 2 mM GlutaMAX (all from Invitrogen) supplemented with 5% (v/v) fetal bovine serum (FBS, Hyclone, Logan, UT), 5 ng/ml FGF2 and 1 ng/ml hTGFβ1 (R&D), at 37°C under 5% CO_2 for up to passage 3.

Scaffold-free cartilage formation: pellet culture.

To induce chondrogenesis from hPSC-derived chondroprogenitors, aliquots of 2-3×10^5 cells were centrifuged in a 15-ml conical tube (BD) to form pellets, and cultured in a 0.5 ml of serum-free chondrogenic media: DMEM (high glucose), 1 mM sodium pyruvate, 1% (v/v) ITS+ (BD),

100 nM dexamethasone (Sigma), 0.17 mM AA2P, 0.35 mM proline, 2 mM GlutaMAX, and 50 μM MTG, supplemented with 40 ng/ml PDGF. On day 6 of pellet culture, 10 ng/ml TGFβ3 was added, and on day 10, a BMP (hBMP4 [PTB condition], hGDF5 [PTG condition], or hBMP7 [PT7 condition], all from R&D) was added at 50 ng/ml. For the dedifferentiated human nasal chondrocytes, chondrogenesis was initiated in the presence of 10 ng/ml TGFβ3 from day 0 as described (Pelttari et al., 2014). In some pellets, 50 ng/ml BMP4 was added on day 10. When necessary the following were added on day 11 or 12: the activator of adenylyl cyclase, forskolin, 3-30 μM (Tocris); canonical WNT signaling inhibitors such as KY02111, 10 μM, (Tocris) (Minami et al., 2012), and β-catenin-TCF interaction inhibitor (Gonsalves et al., 2011), iCRT14, 25 μM, (Tocris); general WNT signaling inhibitors such as the L-type calcium channel blocker that induces soluble WNT binding proteins (Takamatsu et al., 2014), verapamil, 50 μM, (Tocris), and a porcupine inhibitor that inhibits WNT secretion (Proffitt et al., 2013), Wnt-C59, 30-120 nM, (Cellagen Technology, San Diego, CA); and cGMP signaling inhibitors such as the cGMP-dependent protein kinase inhibitor that works at least *in vitro* (Burkhardt et al., 2000), KT5824, 4 μM, (Tocris) and a potent cGMP antagonist (Poppe et al., 2008), Rp-8-Br-PET-cGMPS, 30-60 μM, (Tocris). In separate experiments, the following were added on day 11 or 12: cAMP analogs such as the PKA activator, N^6-benzoyl-cAMP, 40-100 μM, (Tocris and EMD Millipore, Temecula, CA): an EPAC activator (Christensen et al., 2003; Poppe et al., 2008), 8-pCPT-2'-O-Me-cAMP-AM, 3-6 μM, (Tocris and Axxora, Farmingdale, NY); the non-specific phosphodiesterase inhibitor IBMX, 0.5 mM, (Tocris), and non/less-active version of forskolin, 1,9-dideoxyforskolin, 12.5-25 μM, (Tocris). The concentrations used were in a published range that had been successful for various cell-based assays. The pellet cultures were maintained at 37°C under 7.5% CO_2 for up to 40 days. When the cartilage pellet became large enough to fill the bottom of the conical tube (larger than 4 mm in diameter), the pellet was transferred to a well of a 24-well tissue culture-untreated plate (BD), and cultured in 1.0-1.5 ml of chondrogenic medium at 37°C

under 7.5% CO_2/5% O_2. Some cartilage pellets were then fixed with Zinc-Formalin (Z-Fix, American MasterTech, Lodi, CA) for 1 day, paraffin-embedded, sectioned (5 μm), deparaffinized, rehydrated, and stained with 0.1% (w/v) Toluidine Blue (Sigma).

Isolation of chondrocytes from cartilage pellets.

A cartilage pellet was treated with 0.3-0.5 ml of 4 mg/ml collagenase (D [Roche/Sigma]: XI [Sigma]=1:1 in DMEM) (Nakayama et al., 2000) at 37°C for 3 h with occasional shaking. Enzyme digestion was stopped by the addition of 1 ml DMEM, 10% (v/v) FBS. Pellets were dissociated with repetitive pipetting, and remaining aggregates were removed with a 40 μm mesh (BD). Cells were washed twice with an appropriate medium or solution. Approximately 1.5-5.0 x 10^5 cells were recovered per pellet.

EdU labeling of chondrocytes in cartilage pellets.

Ten to 20 μM of EdU (Click-iT Plus EdU AlexaFluor 647 Flow Cytometry Assay Kit, Invitrogen) was added to pellet cultures and maintained for 21-25 h. The labeling time was determined by test labeling of pellets for 3 h, 21 h, 3 days, and 7 days. Under PTB+/-Fk conditions, approximately 1 day of labeling gave the most reliable values of EdU^+ cell population from H9 hESC- and CY2 SOX9-GFP hiPSC-progeny-derived cartilage. Two to three cartilage pellets were combined, and single cells were isolated as described above. Cells were washed with PBS and subjected to brief fixation with para-formaldehyde, followed by permeabilization with saponin, and then to AlexaFluor 647-picolyl azide treatment, according to the manufacturer's recommendation. The labeled cells were analyzed by FACS LSRII (BD).

Isolation and quantification of DNA, RNA and sGAG from cartilage.

Cartilage pellets were collected and submerged in liquid N_2, manually cracked into small pieces with a liquid N_2 cooled mortar and a pellet pestle, and homogenized in the lysis buffer (RLT, Qiagen, Valencia, CA). The cleared lysates were then subjected to DNA, RNA and proteins isolation using the AllPrep DNA/RNA/Protein mini kit (Qiagen). The purified RNA was used for

real-time RT-PCR analysis. The isolated proteins were subjected to papain digestion for 20-24 h at 60^{o}C (125 μg/ml papain, 10 mM cysteine in sodium phosphate-EDTA pH 6.5 [all from Sigma]), and released sGAG was quantified by the 1,9-dimethyl methylene blue (DMMB [Sigma], 16 μg/ml in glycine-NaCl pH 3) serial-dilution assay, using bovine tracheal chondroitin-4-sulfate (Biocolor, UK) as standard. The OD590-530 was measured with SpectraMax M2 (Molecular Devices, Sunnyvale, CA). The DNA isolated with the AllPrep kit was quantified by the Hoechst33258 (Sigma, 0.2 μg/ml in Tris·HCl-EDTA-NaCl pH 7.5) serial-dilution assay, using bovine thymus DNA (Sigma) as standard. The fluorescence (emission 460 nm, excitation 360 nm) was measured with SpectraMax M2. The total sGAG and DNA amounts per particle, along with comparative ratios of the sGAG and DNA, were then calculated. Results are presented as mean values with SEM (the standard error of the mean) shown by thin error lines.

Gene expression profiling.

The isolated RNA was reverse transcribed (RT) using a Superscript III kit (Invitrogen) and real-time polymerase chain reaction (RT-PCR) was performed using the Taqman Gene Expression Assay and ABI7900 (Applied Biosystems, Foster City, CA). The expression levels of individual genes from duplicate or triplicate reactions were normalized against *EEF1A1* transcript ($2^{-\Delta Ct}$ x100) and averaged to obtain relative expression, as described (Wang and Nakayama, 2009). The RT-PCR results are presented as mean relative expression levels with SEM shown by the thin error lines. Undetectable levels of all the genes tested lie in the relative expression range of 0.001 to 0.0001. The "*% COL10A1/COL2A1"* is a relative expression of *COL10A1* normalized against that of *COL2A1* and multiplied by 100. The "Change in expression" was determined by the ratio of gene expression levels between treated (+) and untreated (-) pellets.

For RNA Sequencing analysis (RNA-seq), three independent sets of PTB pellets, and four independent sets of PTBFk pellets were prepared from passage 5-8 ectomesenchymal cells (i.e., FSb-expanded CD271^{hi}CD73^{-} neural crest-like progeny of H9 hESCs). On day 26-28

of pellet culture, 5 to 6 cartilage pellets were harvested, combined and total RNA was extracted with an RNeasy mini kit (Qiagen). Poly (A)-tailed messenger RNA was enriched using Poly(A)Purist Kit (Ambion, Foster City, CA) before the preparation of the RNA-seq library using an Ultra directional RNA library prep kit for Illumina (New England Biolabs, Ipswich, MA) per manufacturer's instructions. RNA-seq was performed using the Illumina Nextseq500 with the 150 bp pair-ended running mode. Sequencing reads were aligned against the GRCh37/hg19 reference genome using bowtie2 (Langmead and Salzberg, 2012) with default parameter. Only uniquely mapped reads were used for downstream analysis. HTseq was used to count the read numbers mapped to each gene. DESeq2 (Love et al., 2014) was used to call the significantly differentially expressed genes (fold change ≥2; false discovery rate ≤0.05) between two conditions. The normalized gene counts from DESeq2 was used to do the normalization. The analyzed data are summarized in Tables S1-4. Sequenced reads were deposited to GEO (Accession #: GSE116173).

Subcutaneous transplantation of cartilage particles.

In preparation for cartilage transplantation, 6-12-week-old female immunocompromized NSG mice (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ; Stock No: 005557) were anesthetized with isoflurane. After they had lost the pedal withdrawal reflex, buprenorphine was injected subcutaneously near the proposed site of incision, followed by clipping of back hair, skin disinfection with chlorhexidine and 70% (v/v) alcohol, and placement of a sterile drape around the area of incision. Mice were placed on a heated pad during the procedure to preserve body temperature. Two mid-longitudinal skin incisions of approximately 1 cm were made on the dorsal neck area of each mouse, and subcutaneous pockets formed by blunt dissection. *In vitro*-made cartilage pellets (of approximately 1-5 mm "wet" diameter) were individually placed into each pocket, with up to two transplants per mouse. Incisions were closed with skin adhesive. After 8 weeks, the transplanted mice were euthanized and cartilage pellets were harvested, fixed with Z-Fix for 4 days, embedded in plastic, sectioned (5 μm), deplastified, rehydrated and stained with von

Kossa counterstained with van Gieson, or with Toluidine Blue. Control experiments were performed using a piece of articular cartilage surface of 2-year-old bovine knee (4 mm in diameter x 2-3 mm in depth) obtained from Animal Technologies (Tyler, TX). The transplantation experiments were performed under the regulation of IACUC for the University of Texas Health Science Center at Houston (UTHealth).

Immunohistological staining.

The *in vitro*-made cartilage pellets were fixed with Z-Fix for 1 day, and paraffin embedded, sectioned (5 μm), deparaffinized with xylene, rehydrated, heat treated in the antigen-retrieval solution (Dako, Glostrup, Denmark), blocked with the blocking buffer (Dako), and subjected to immunofluorescence detection of COL1 (for detecting mesenchymal cells or osteoblasts), COL2 (for detecting chondrocytes), or Ki67 (for detecting proliferating cells). The sections of *in vivo*-derived, plastic-embedded cartilage pellets were deplastified in 1-acetoxy-2-methoxyethane (Sigma) for 30 min, rehydrated, DeCal (BioGenex Lab, Fremont, CA) and heat treated, blocked, and subjected to immunofluorescence detection of COL2 or COL10 (for detecting hypertrophic chondrocytes). Primary antibodies were rabbit anti-COL1 antibody (#NB600-408) and biotinylated goat anti-COL2 antibody (#NBP1-26546) from Novus Biologicals (Littleton, CO), and rabbit anti-human Ki67 antibody (#AB66155) and rabbit anti-COL10 antibody (#AB58632) from Abcam (Cambridge, MA). Secondary reagents were goat anti-rabbit IgG-AlexaFluor488 (#A11034) and streptavidin-AlexaFluor594 (#S11227) from Molecular Probes (Eugene, OR). The slides were washed in PBS and mounted with ProLong Gold anti-fade mounting media (Molecular Probes). The % $Ki67^+$ nuclei/$DAPI^+$ nuclei of the $COL2^+$ area was calculated as the average ratio between the number of $Ki67^+$ nuclei and that of ($DAPI^+$) nuclei counted from 3-4 different $COL2^+$ areas per section and multiplied by 100.

Flow Cytometry.

FACS analysis was performed on LSR II (BD). Cell sorting was done with FACS Aria II (BD) as described (Umeda et al., 2015; Umeda et al., 2012). Viable single cells were gated using DAPI (Sigma). Sorted cells ranged in purity from 90 to 95%.

Statistical Analysis.

Statistical differences between groups were determined by Student's t-test (2 categories) or 1-way ANOVA (>2 categories) followed by the Student-Newman-Keuls multiple comparisons by KaleidaGraph (Synergy, Reading, PA) software. n = number of independent experiments.

*: $P<0.1$, **: $P<0.05$, ***: $P<0.01$. $P<0.05$ is considered to be statistically significant, and $P<0.1$ is weakly significant.

4. SUPPLEMENTAL REFERENCES

Burkhardt, M., Glazova, M., Gambaryan, S., Vollkommer, T., Butt, E., Bader, B., Heermeier, K., Lincoln, T.M., Walter, U., and Palmetshofer, A. (2000). KT5823 inhibits cGMP-dependent protein kinase activity in vitro but not in intact human platelets and rat mesangial cells. J Biol Chem *275*, 33536-33541.

Centola, M., Abbruzzese, F., Scotti, C., Barbero, A., Vadala, G., Denaro, V., Martin, I., Trombetta, M., Rainer, A., and Marsano, A. (2013). Scaffold-based delivery of a clinically relevant anti-angiogenic drug promotes the formation of in vivo stable cartilage. Tissue Eng Part A *19*, 1960-1971.

Christensen, A.E., Selheim, F., de Rooij, J., Dremier, S., Schwede, F., Dao, K.K., Martinez, A., Maenhaut, C., Bos, J.L., Genieser, H.G.*, et al.* (2003). cAMP analog mapping of Epac1 and cAMP kinase. Discriminating analogs demonstrate that Epac and cAMP kinase act synergistically to promote PC-12 cell neurite extension. J Biol Chem *278*, 35394-35402.

Gonsalves, F.C., Klein, K., Carson, B.B., Katz, S., Ekas, L.A., Evans, S., Nagourney, R., Cardozo, T., Brown, A.M., and DasGupta, R. (2011). An RNAi-based chemical genetic screen identifies three small-molecule inhibitors of the Wnt/wingless signaling pathway. Proc Natl Acad Sci U S A *108*, 5954-5963.

Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. Nat Methods *9*, 357-359.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol *15*, 550.

Minami, I., Yamada, K., Otsuji, T.G., Yamamoto, T., Shen, Y., Otsuka, S., Kadota, S., Morone, N., Barve, M., Asai, Y.*, et al.* (2012). A small molecule that promotes cardiac differentiation of human pluripotent stem cells under defined, cytokine- and xeno-free conditions. Cell Rep *2*, 1448-1460.

Nakayama, N., Lee, J., and Chiu, L. (2000). Vascular endothelial growth factor synergistically enhances bone morphogenetic protein-4-dependent lymphohematopoietic cell generation from embryonic stem cells in vitro. Blood *95*, 2275-2283.

Pelttari, K., Pippenger, B., Mumme, M., Feliciano, S., Scotti, C., Mainil-Varlet, P., Procino, A., von Rechenberg, B., Schwamborn, T., Jakob, M.*, et al.* (2014). Adult human neural crest-derived cells for articular cartilage repair. Sci Transl Med *6*, 251ra119.

Poppe, H., Rybalkin, S.D., Rehmann, H., Hinds, T.R., Tang, X.B., Christensen, A.E., Schwede, F., Genieser, H.G., Bos, J.L., Doskeland, S.O.*, et al.* (2008). Cyclic nucleotide analogs as probes of signaling pathways. Nat Methods *5*, 277-278.

Proffitt, K.D., Madan, B., Ke, Z., Pendharkar, V., Ding, L., Lee, M.A., Hannoush, R.N., and Virshup, D.M. (2013). Pharmacological inhibition of the Wnt acyltransferase PORCN prevents growth of WNT-driven mammary cancer. Cancer Res *73*, 502-507.

Takamatsu, A., Ohkawara, B., Ito, M., Masuda, A., Sakai, T., Ishiguro, N., and Ohno, K. (2014). Verapamil protects against cartilage degradation in osteoarthritis by inhibiting Wnt/beta-catenin signaling. PLoS One *9*, e92699.

Umeda, K., Oda, H., Yan, Q., Matthias, N., Zhao, J., Davis, B.R., and Nakayama, N. (2015). Long-Term Expandable SOX9(+) Chondrogenic Ectomesenchymal Cells from Human Pluripotent Stem Cells. Stem Cell Reports *4*, 712-726.

Umeda, K., Zhao, J., Simmons, P., Stanley, E., Elefanty, A., and Nakayama, N. (2012). Human chondrogenic paraxial mesoderm, directed specification and prospective isolation from pluripotent stem cells. Sci Rep *2*, 455.

Wang, Y., and Nakayama, N. (2009). WNT and BMP signaling are both required for hematopoietic cell development from human ES cells. Stem Cell Res *3*, 113-125.

Wang, Y., Umeda, K., and Nakayama, N. (2010). Collaboration between WNT and BMP signaling promotes hemoangiogenic cell development from human fibroblast-derived iPS cells. Stem Cell Res *4*, 223-231.