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Supplemental Information

Induced Pluripotent Stem Cells to Model Human Fibrodysplasia Ossificans Progressiva

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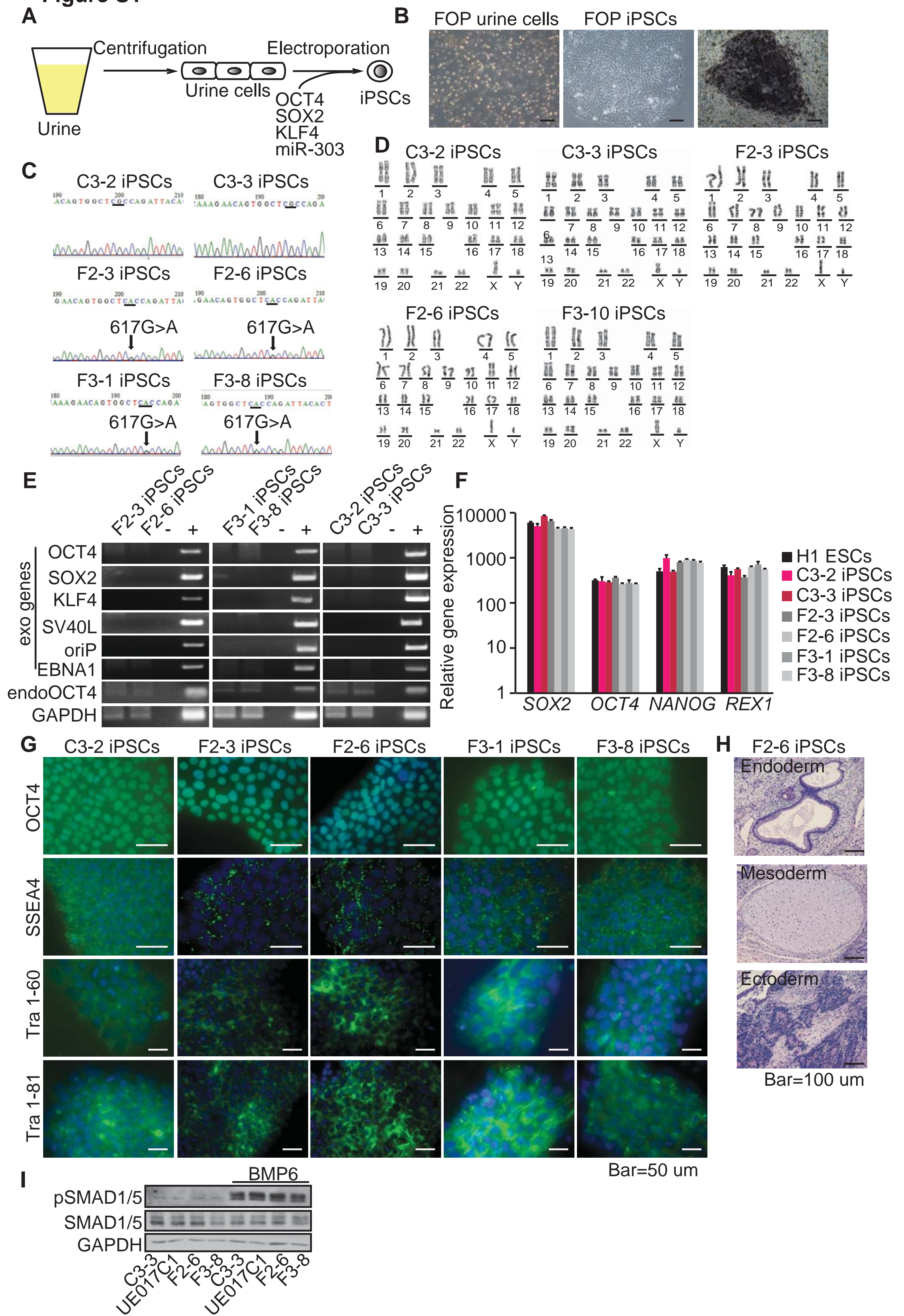

Figure S1
A
Centrifugation
Electroporation
Urine cells
OCT4
SOX2
KLF4
miR-303
iPSCs
Urine
B
FOP urine cells
FOP iPSCs
C
C3-2 iPSCs
C3-3 iPSCs
F2-3 iPSCs
F2-6 iPSCs
617G>A
F3-1 iPSCs
F3-8 iPSCs
D
C3-2 iPSCs
C3-3 iPSCs
F2-3 iPSCs
F2-6 iPSCs
F3-10 iPSCs
E
F2-3 iPSCs
F2-6 iPSCs
F3-1 iPSCs
F3-8 iPSCs
C3-2 iPSCs
C3-3 iPSCs
exo genes
OCT4
SOX2
KLF4
SV40L
oriP
EBNA1
endoOCT4
GAPDH
F
Relative gene expression
H1 ESCs
C3-2 iPSCs
C3-3 iPSCs
F2-3 iPSCs
F2-6 iPSCs
F3-1 iPSCs
F3-8 iPSCs
SOX2
OCT4
NANOG
REX1
G
OCT4
SSEA4
Tra 1-60
Tra 1-81
Bar=50 um
H
F2-6 iPSCs
Endoderm
Mesoderm
Ectoderm
Bar=100 um
I
BMP6
pSMAD1/5
SMAD1/5
GAPDH
C3-3
UE017C1
F2-6
F3-8

Figure S2

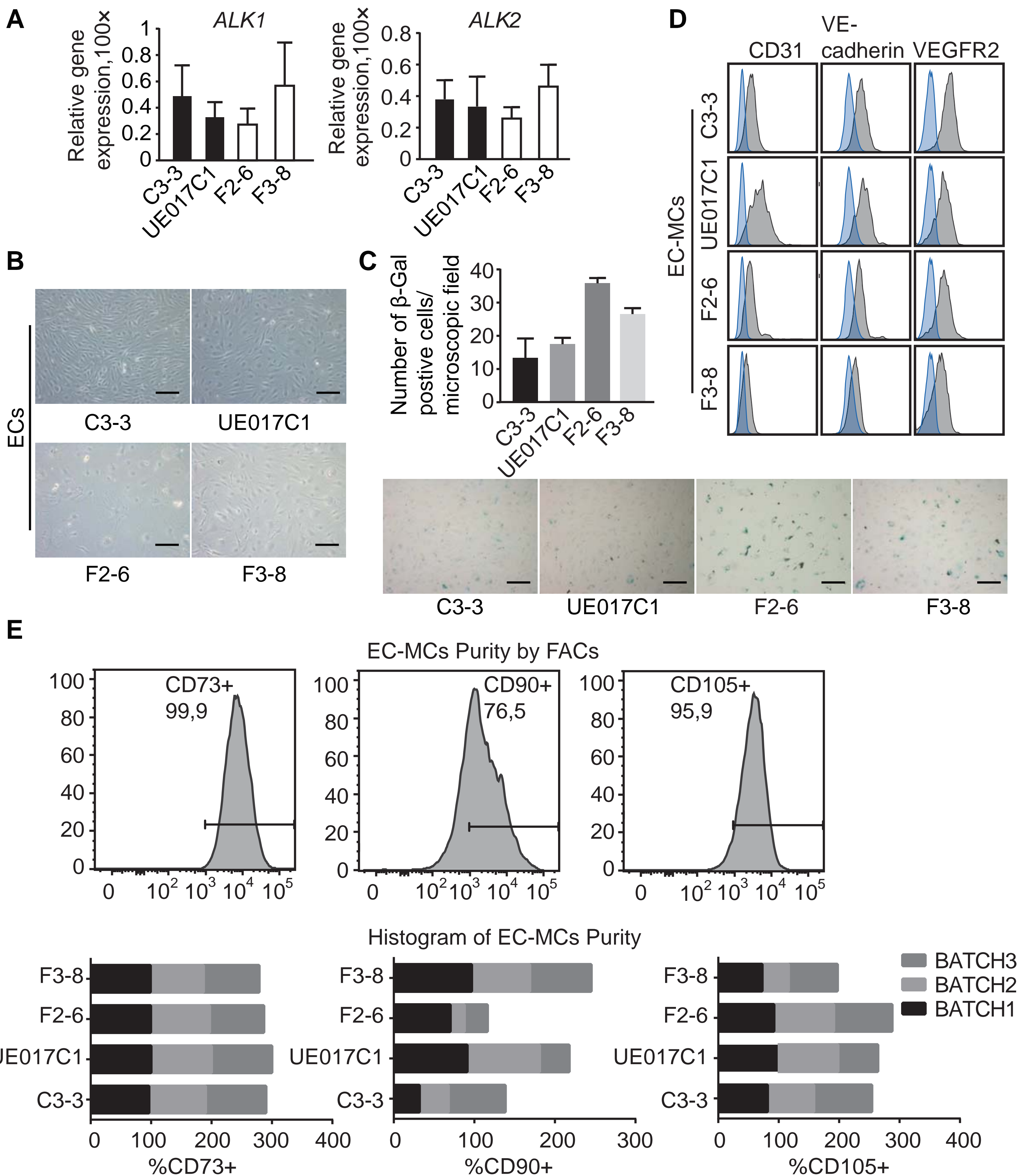

Figure S3

A

Pericytes Purity by FACs

CD73+ 99,9

CD90+ 99,2

CD105+ 100,0

PDGFRβ+ 97,0

CD146+ 100

NG2+ 99,2

Histogram of Pericytes Purity

BATCH5
BATCH4
BATCH3
BATCH2

%CD73+

%CD90+

%CD105+

%PDGFRβ+

%CD146+

%NG2+

B

ALK2

Relative gene expression,100×

C3-3
UE017C1
F2-6
F3-8

Figure S1. Characterization of hiPSCs from FOP Patients and Healthy Donor

(A) Schematic representation of the reprogramming of hiPSCs from urine cells.

(B) Left panel, bright field image of cultured patient F2 urine cells. Middle panel, bright field image of cultured human FOP hiPSCs. Right panel: ALP staining of FOP hiPSCs. Scale bars, 250 μm.

(C) Sequencing results of the G→A mutation site in the FOP hiPSCs.

(D) Normal karyotypes of FOP hiPSCs and control hiPSCs.

(E) Lack of vector DNA in expanded hiPSCs that have been passaged for >10 times. PCR analysis on total DNA extracted from control and FOP hiPSCs was performed by using primers that specifically recognize exogenous transgenes (exo genes, OCT4, SOX2, KLF4, SV40L, oriP and EBNA1). Endogenous OCT4 (endoOCT4) is used for the detection of OCT4 expression and GAPDH was used as the loading control. Positive control, genome DNA extracted from urine cells that were transfected with related episome vectors. Negative control, water.

(F) qPCR analysis of endogenous *OCT4*, *SOX2*, *NANOG*, and *REX1* mRNA expression in FOP hiPSCs and H1 ESCs. H1 ESCs were used as positive control. *ACTIN* was used to normalize gene expression. Each value is the means ± SD. All the values were adjusted to urine cells value, which is defined as 1. The values represent the average level of 3 independent samples.

(G) Immunofluorescence staining of hiPSCs specific cell markers. Green: antibody indicated in the figure; blue: DAPI. Scale bars, 50 μm.

(H) Hematoxylin/eosin staining of teratoma derived from FOP hiPSCs (F2-6). Scale bars, 100 μm.

(I) Western blot in FOP and control hiPSCs. After serum starvation for 6 hours, hiPSCs were stimulated with or without BMP6 (5ng/ml) for 1 hour. Protein was isolated and western

blotting was performed to check the SMAD1/5 phosphorylation. GAPDH was used as loading control; all the experiments represented 2 independent biological replicates.

Figure S2: hiPSCs-ECs and EC-MCs Characterization. Related to Figure 2.

(A) The mRNA expression level of *ALK1* and *ALK2* in $CD31^+$ ECs. Each value is the means ± SD. All the experiments represented 3 independent biological replicates and were normalized to *ACTIN*.

(B) Bright field image of ECs on passage 3 after cell sorting. Scale bars, 100 μm.

(C) Upper panel: Numbers of β-galactosidase positive cells per microscopic field. Lower panel: Bright field image of β-galactosidase staining of ECs. Scale bars, 100 μm. All the experiments were repeated 3 times, and one of the representative results is shown. Values are presented as mean and SD from 3 technique replicates.

(D) FACs analysis for ECs markers in EC-MCs. Blue, unstained cells; grey, antibody as indicated.

(E) Upper panel: FACs gates illustrating percentage of CD73, CD90 and CD105 positive cells. Lower panel: Histogram of CD73, CD90 and CD105 positive cells analyzed in this study from 3 batches of experiments, the average data from 3 batches of experiments showed in Figure 2F.

Figure S3: hiPSC-pericytes Characterization. Related to Figure 3.

(A) Upper part: FACs gates illustrating percentage of CD73, CD90, CD105, PDGFRβ, CD146 and NG2 positive cells. Lower part: Histogram of CD73, CD90, CD105, PDGFRβ, CD146 and NG2 positive cells analyzed in this study from 4 batches of experiments, the average data from 4 batches of experiments showed in Figure 3A.

(B) The mRNA expression level of *ALK2* in $CD31^-$ pericytes. Each value is the means ± SD. All the experiments represented 3 independent biological replicates and were normalized to *ACTIN*.

Supplemental Experimental Procedures

Ethical Statement

The participants in this manuscript have signed written informed consent for donating human urine cells for stem cell generation. Experiments involved with human subjects was approved by IRB GIBH-IRB02-2009002 at Guangzhou Institutes of Biomedicine and Health (GIBH), and 12/467 (2013 January) at VU university medical center. The animal research was approved by IRB at GIBH (NO. 2010012).

Urine Cell Culture and hiPSCs Generation

Urine cells were collected and maintained as previously publication (Zhou et al., 2011). Urine cells within 5 passages were used for iPS cells generation. hiPSC lines were generated from 5×10^5–1×10^6 urine cells which was electroporated with episomal vectors (3.5 μg pEP4EO2SET2K (contains *OCT4*, *SOX2*, *SV40LT* and *KLF4*) and pCEP4-miR-302-367 cluster (contains *miR-302b, c, a, d* and *miR-367*) by Amaxa™ Basic Nucleofector™ kit for primary mammalian epithelial cells, program T-020 (LONZA). The transfected urine cells were immediately seeded onto matrigel (BD Biosciences, 354230) pre-coated 6-well plates. Refreshed the medium with defined medium mTeSR1 (StemCell Technologies, 05852) with TGFβ/Activin/Nodal receptor inhibitor A-83-01 (Sigma, SML0788), MEK inhibitor PD0325901, GSK3b inhibitor CHIR99021 and ROCK inhibitor thiazovivin (gifts from Dr. Ke Ding) the next day and changed medium every other day. The appeared hiPSCs were passaged by mechanical picking and replated in matrigel pre-coated plates between day 20 and day 30 after induction. The hiPSCs were routinely maintained in mTesR1 medium and passaged by dispase (1 mg/ml in DMEM/F12, Gibco, 17105-041) or by 0.5 mM EDTA (Invitrogen, AM9260G). H1 human embryonic stem cells brought from WiCell Research Institute (Madison, WI). UE017C1 (uiPSC-015 C1 in the paper) was obtained from the Guangzhou Stem Cell Bank and was previously characterized (Xue et al., 2013).

hiPSCs Characterization

qPCR, transgene integration, immunofluorescence (OCT-3/4 Antibody, Santa Cruz Biotechnology sc-5279; Human NANOG Affinity Purified Polyclonal Ab, R&D AF1997; Anti-SSEA4 antibody, Abcam AB16287; Anti-TRA-1-60, Millipore MAB 4360; Anti-TRA-1-81, Millipore MAB 4381) and karyotyping were done as described (Xue et al., 2013); the primers used for transgene integration and qPCR also published before (Xue et al., 2013). For teratomas, hiPSCs were resuspended with 30% matrigel and then injected subcutaneously and intramuscularly into the flanks of SCID mice. Tumors were sectioned after 7 weeks and stained with hematoxylin/eosin.

ALK2 Mutational Analysis

Genomic DNA of hiPSCs was isolated by using Wizard® Genomic DNA Purification Kit (Promega, A1120), PCR reaction was conducted as previous publication using primers as following (Kaplan et al., 2008): Forward primer: 5'-CCAGTCCTTCTTCCTTCTTCC-3', Reverse primer: 5'-AGCAGATTTTCCAAGTTCCATC-3'. PCR products were sequenced to identify the mutation sites.

Differentiation hiPSCs into ECs and Pericytes

ECs and pericytes differentiation was initiated by culture hiPSCs in basal medium BEPL and growth factors Activin A (25ng/ml, R&D, 338-AC-010), BMP4 (30ng/ml, R&D, 314-BP-010/CF), VEGF (30ng/ml, R&D Systems, 293-VE) and the small molecule inhibitor CHIR99021 (1.5µM, Tocris, 4423) for 3 days. The medium was refreshed with BPEL with VEGF and SB43152 (10µM, Tocris, 1614) from day 4 to day 10 of differentiation. ECs were isolated by $CD31^-$ labeled Dynabeads (Life Technologies, 11155D). Sorted $CD31^+$ ECs were maintained in human endothelial growth (serum free) medium (hEC-SFM) (Life Technologies, 11111) supplemented with 1% platelet poor plasma (BTI, BT-214), 30ng/ml VEGF and basic fibroblast growth factor (bFGF) (20ng/ml, R&D, 100-18B) on 0.1% gelatin

(Sigma-Aldrich, G1890) coated plates, and passaged every 3-4 days by TrypLE Select (Gibco, 12563-011). $CD31^-$ cells were plated in EGM-2 media (Lonza, CC-3162). Medium changed to DMEM+10% FBS (Gibco, 10270), supplemented with or without TGFβ3 (1ng/ml, a generous gift of Kenneth K. Iwata, OSI Pharmaceuticals) and PDGF-BB (4ng/ml, Peprotech, 100-14B) for 1 day when cells were confluent. Afterwards, the hiPSCs derived pericytes were routinely maintained in DMEM+10% FBS on gelatin pre-coated plates.

Flow Cytometry (FACs) Analysis

Cells were dissociated by TrypLE Select and washed with the FACs buffer with 10% FBS, followed the other time of washing by the FACs buffer. The following antibodies was used for the FACs staining: VE-cadherin-A488 (eBiosciences, 53-1449-41, 1:100), CD31-APC (eBiosciences, 17-0319, 1:200), KDR-PE (R&D Systems, FAB357P, 1:50), PDGFRβ-PE (BD Pharmingen, 558821, 1:50), CD73-PE (BD Pharmingen, 550257, 1:50), CD105-PE (Life Technologies, MHCD10504, 1:200), CD90-PE (1:400), NG2-PE (R&D, FAB2585P, 1:50), CD146-FITC (BD, P1H12, 1:50). Samples were analyzed with the MACSQuant VYB (Miltenyi) with the following instrument settings Blue/488 FITC, A488: 525/50, Yellow/561 PE: 586/15, APC: 661/20. Unstained cells were used as negative controls for FACS gating. FACS data were analyzed using FlowJo 10.1 software.

Cell senescence assay

β-galactosidase (β-gal) activity was performed using a senescence detection kit (Cell Signaling, 9860S) according to the manufacturer's instructions. The number of β-gal positive cells was counted in randomly selected microscopic fields (magnification ×10).

Alkaline Phosphatase (ALP) Assay

Pericytes were seeded in 96 well plate pre-coated with 0.1% gelatin. After pretreating LDN-212854 (gift from Paul Yu) for 1 day, differentiation of pericytes was initiated in osteogenic medium, which is comprised of α-MEM (Gibco, 32561-029) supplemented with 10% FBS,

0.2 mM ascorbic acid (Sigma, A8960), dexamethasone (Sigma, D4902) and 10 mM of β-glycerophosphate (Sigma, G6251), medium refreshed every 3-4 days. Histochemical examination of ALP activity was performed using naphtol AS-MX phosphate (Sigma, N4875) and fast blue RR salt (Sigma, F0500), as described previously (Shi et al., 2013).

Chondrogenic Differentiation

Chondrocytes differentiation was performed according to previous publication (Greco et al., 2011). Briefly, hiPSC-pericytes were suspended in growth medium at a density of 1.5×10^7/ml, micromasses were seeded in 48-well plates by pipetting 10 μl of cell suspension into each wells. The droplets were leave in the incubator for 3 hours for attachment, after that the growth medium was added. The growth medium was changed to chondrogenic medium (Dulbecco's modified Eagle's medium/F-12 (Invitrogen), 1% (v/v) ITS+ Premix (Corning), 50 μg/ml ascorbic acid, 40 μg/ml L-Proline (Sigma), 0.1 mM dexamethasone, 100 μg/ml sodium pyruvate) 24 hours later and culture for another 3 days before stained with 1% (w/v) Alcian Blue (pH 1.0, Sigma). The quantification of Alcian blue staining was performed by incubated stained micromass with 125 μl 6 M guanidine hydrochloride overnight. The absorbance of the Alcian blue solution was measured at 595 nm to determine the relative amount of bound GAGs in the micromass.

Mineralization assay

The mineralization assay was performed after subsequent 3 weeks of culturing in osteogenic medium. To visualize mineralization, cells were stained with 2% alizarin red S solution (Sigma, A5533).

Western Blotting

Cell lysate was isolated from subconfluent cells cultured for 24 hours in α-MEM+1% FBS. Western blotting was performed as previously described using standard techniques (Shi et al., 2013). The antibodies used for immunoblotting were phosphorylated SMAD1/5 antibody

(1:1000, Cell signaling Technology, Danvers, MA, USA, 9511), SMAD1/5 antibody (1:1000, Santa Cruz, SC-6031) and GAPDH antibody (1:40,000, Sigma). Protein expression was quantification of by ImageJ software (NIH). GAPDH was used as the loading control.

Statistical Analyses

Error bars indicated standard deviation of the mean. We treated each cell line as an individual biological replicate and pooled our results into control group (C3-3 and UE017C1) or FOP group (F2-6 and F3-8). At least three replicates of each of the two control or two FOP hiPSCs or derived cells were performed. Differences between the control group and the FOP group were evaluated by t-test or one-way ANOVA with Tukey's multiple comparison tests by using the Prism Software (6.01 version; GraphPad Software). Differences were considered significance when $p < 0.05$.

qPCR primers used in this study

Markers	Forward primer (5'-3')	Reverse primer (5'-3')
ID1	CTGCTCTACGACATGAACGG	GAAGGTCCCTGATGTAGTCGAT
ID3	CACCTCCAGAACGCAGGTGCTG	AGGGCGAAGTTGGGGCCCAT
ALP	GACCCTTGACCCCCACAAT	GCTCGTACTGCATGTCCCCT
COL1α	CAGCCGCTTCACCTACAGC	TTTGTATTCAATCACTGTCTTGCC
OSC	GAAGCCCAGCGGTGCA	CACTACCTCGCTGCCCTCC
ALK1	ATGACCTCCCGCAACTCGA	TAGAGGGAGCCGTGCTCGT
ALK2	TGCCTTCGAATAGTGCTGTC	CATCAAGCTGATTGGTGCTC
OCT4	ACGACCATCTGCCGCTTTG	GCTTCCTCCACCCACTTCTG
NANOG	GCCGAAGAATAGCAATGGTG	TGGTGGTAGGAAGAGTAGAGG
T	ATCACCAGCCACTGCTTC	GGGTTCCTCCATCATCTCTT
PDGFRα	ATTGCGGAATAACATCGGAG	GCTCAGCCCTGTGAGAAGAC
ETV2	CAGCTCTCACCGTTTGCTC	AGGAACTGCCACAGCTGAAT
CD31	GCATCGTGGTCAACATAACAGAA	GATGGAGCAGGACAGGTTCAG
CD105	CCCGCACCGATCCAGACCACTCCT	TGTCACCCCTGTCCTCTGCCTCAC
PDGFRβ	ACGGAGAGTGTGAATGACCA	GATGCAGCTCAGCAAATTGT
NG2	CCAGGAAAGGCAACCTTCAAC	ACGGAAACGGAAGGTGTCC
ACTIN	AATGTCGCGGAGGACTTTGATTGC	AGGATGGCAAGGGACTTCCTGTA A

PCR Transgene primers

Markers		(5'-3')
OCT4	Oct4-SF1	AGTGAGAGGCAACCTGGAGA
	IRES2-SR	AGGAACTGCTTCCTTCACGA
SOX2	Sox2-SF1	ACCAGCTCGCAGACCTACAT
	SV40pA-R	CCCCCTGAACCTGAAACATA
KLF4	Klf4-SF1	CCCACACAGGTGAGAAACCT
	SV40pA-R	CCCCCTGAACCTGAAACATA
SV40L	SV40T-SF1	TGGGGAGAAGAACATGGAAG
	IRES2-SR	AGGAACTGCTTCCTTCACGA
oriP	pEP4-SF1	TTCCACGAGGGTAGTGAACC
	pEP4-SR1	TCGGGGGTGTTAGAGACAAC
EBNA1	pEP4-SF2	ATCGTCAAAGCTGCACACAG
	pEP4-SR2	CCCAGGAGTCCCAGTAGTCA
endoOCT4	endoOct4-F2	AGTTTGTGCCAGGGTTTTTG
	endoOct4-R2	ACTTCACCTTCCCTCCAACC
GAPDH	GAPDH-F	GTGGACCTGACCTGCCGTCT
	GAPDH-R	GGAGGAGTGGGTGTCGCTGT

Supplemental References

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