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Supplemental Information

SMAD3 mediates the specification of human induced pluripotent stem cell-derived epicardium into progenitors for the cardiac pericyte lineage

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SUPPLEMENTAL INFORMATION

Figure S1

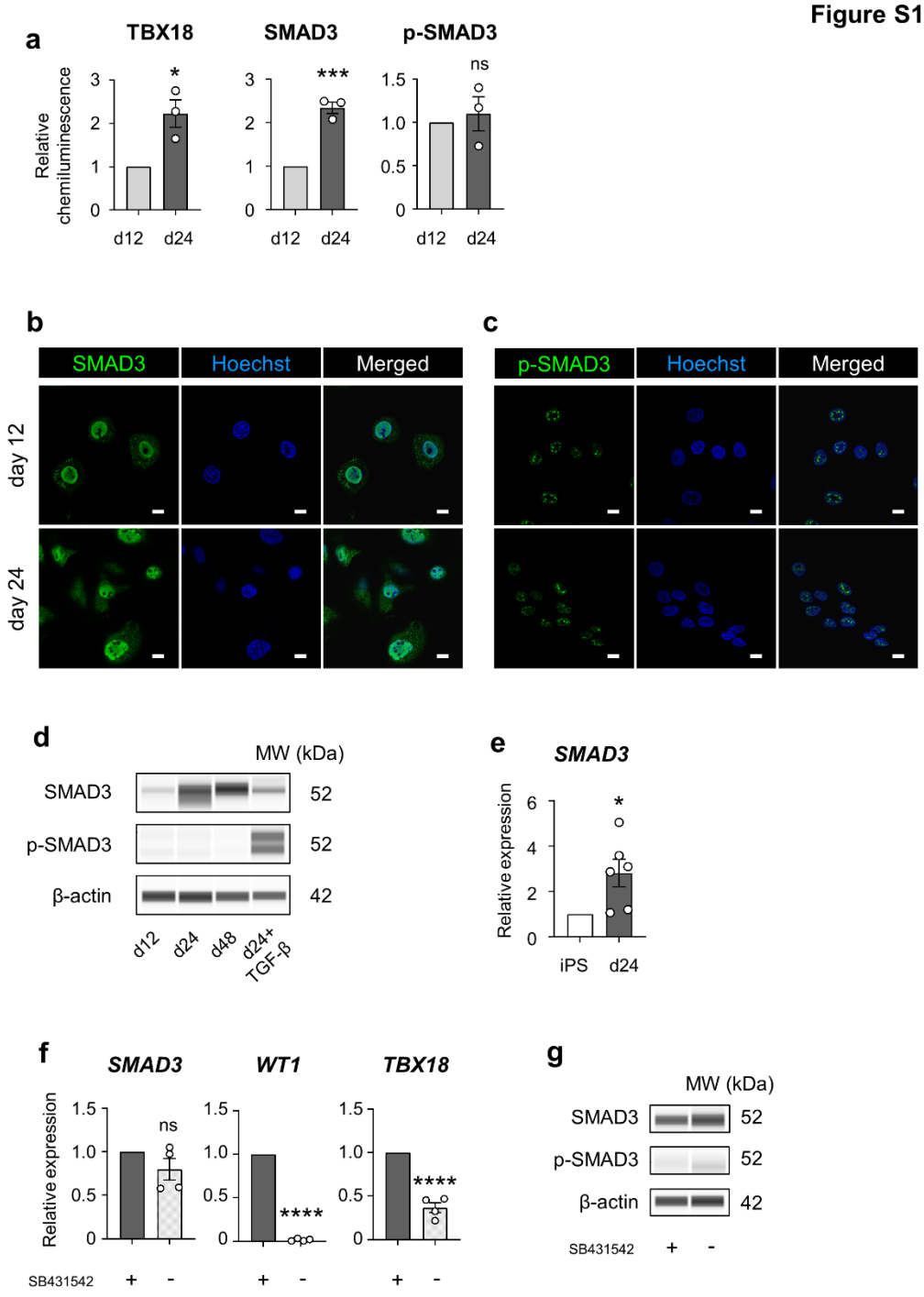

Figure S2

Figure S2

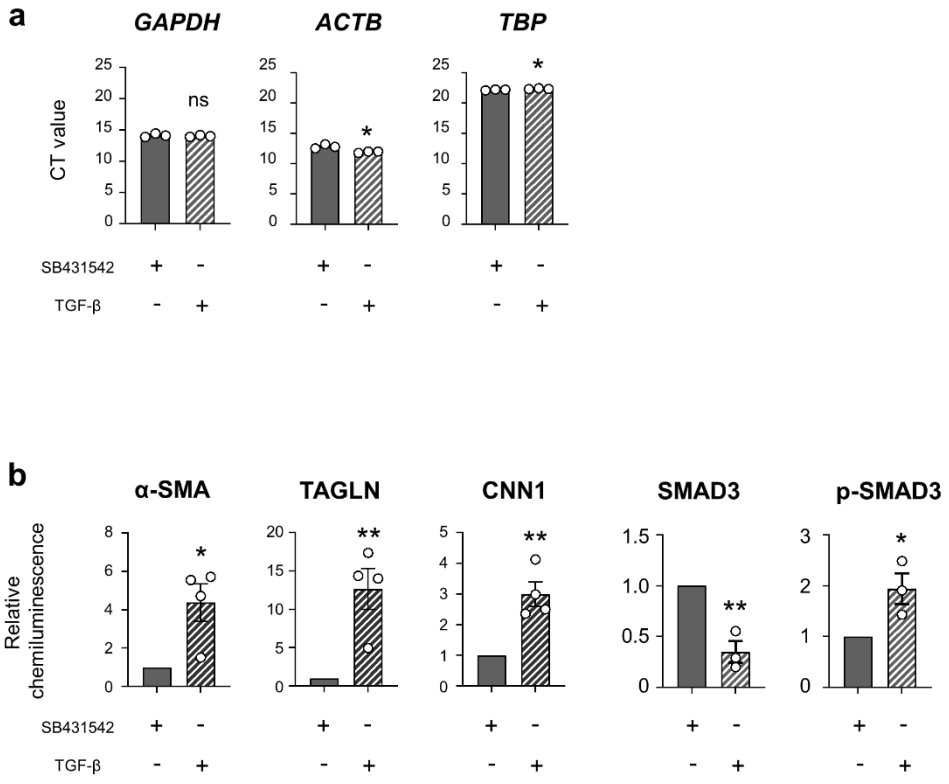

Figure S3

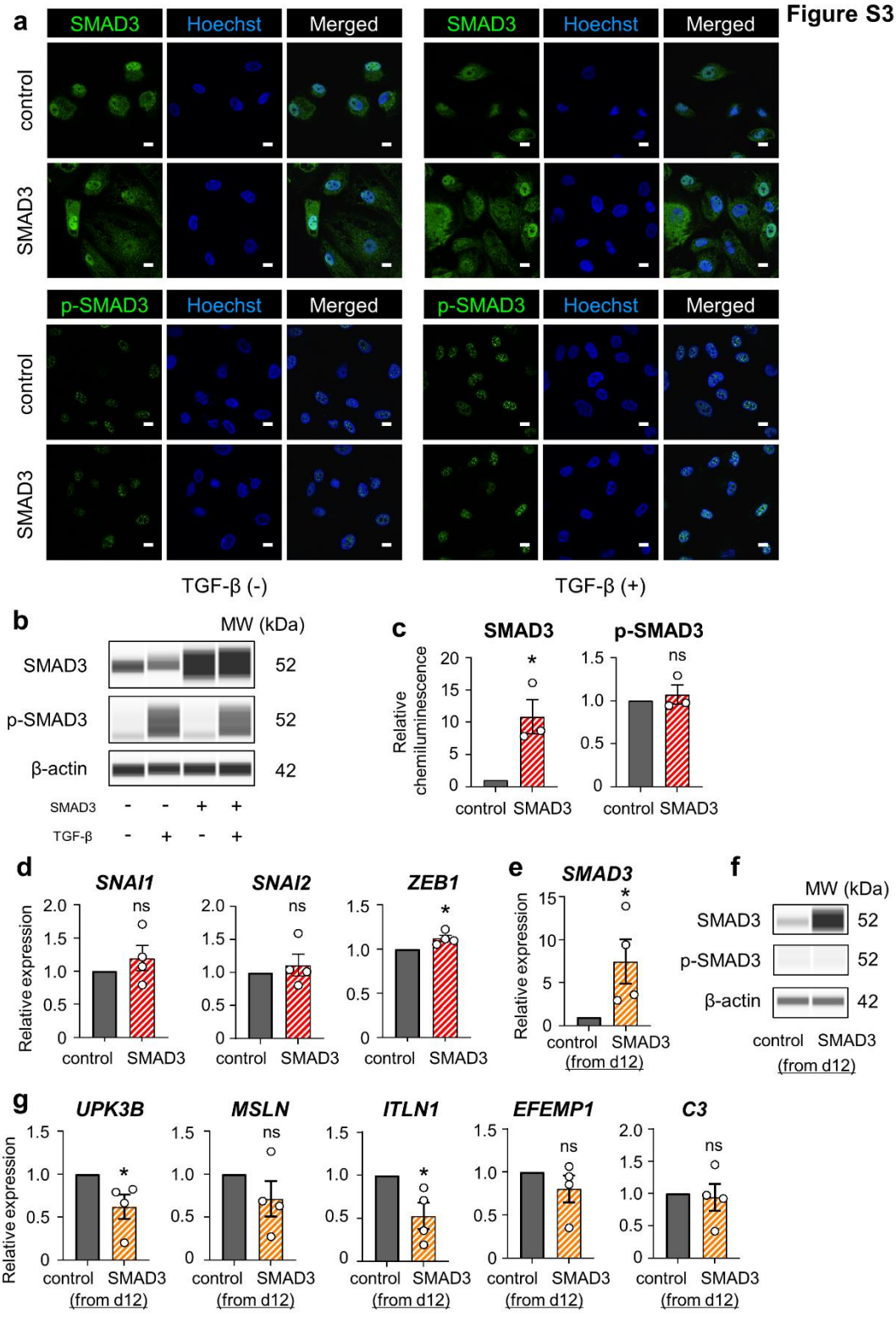
Figure S3
a
SMAD3
Hoechst
Merged
control
SMAD3
p-SMAD3
TGF-β (-)
TGF-β (+)
b
MW (kDa)
SMAD3
p-SMAD3
β-actin
52
42
TGF-β
c
SMAD3
p-SMAD3
Relative chemiluminescence
ns
control
d
SNAI1
SNAI2
ZEB1
Relative expression
e
SMAD3
(from d12)
f
g
UPK3B
MSLN
ITLN1
EFEMP1
C3

Figure S4

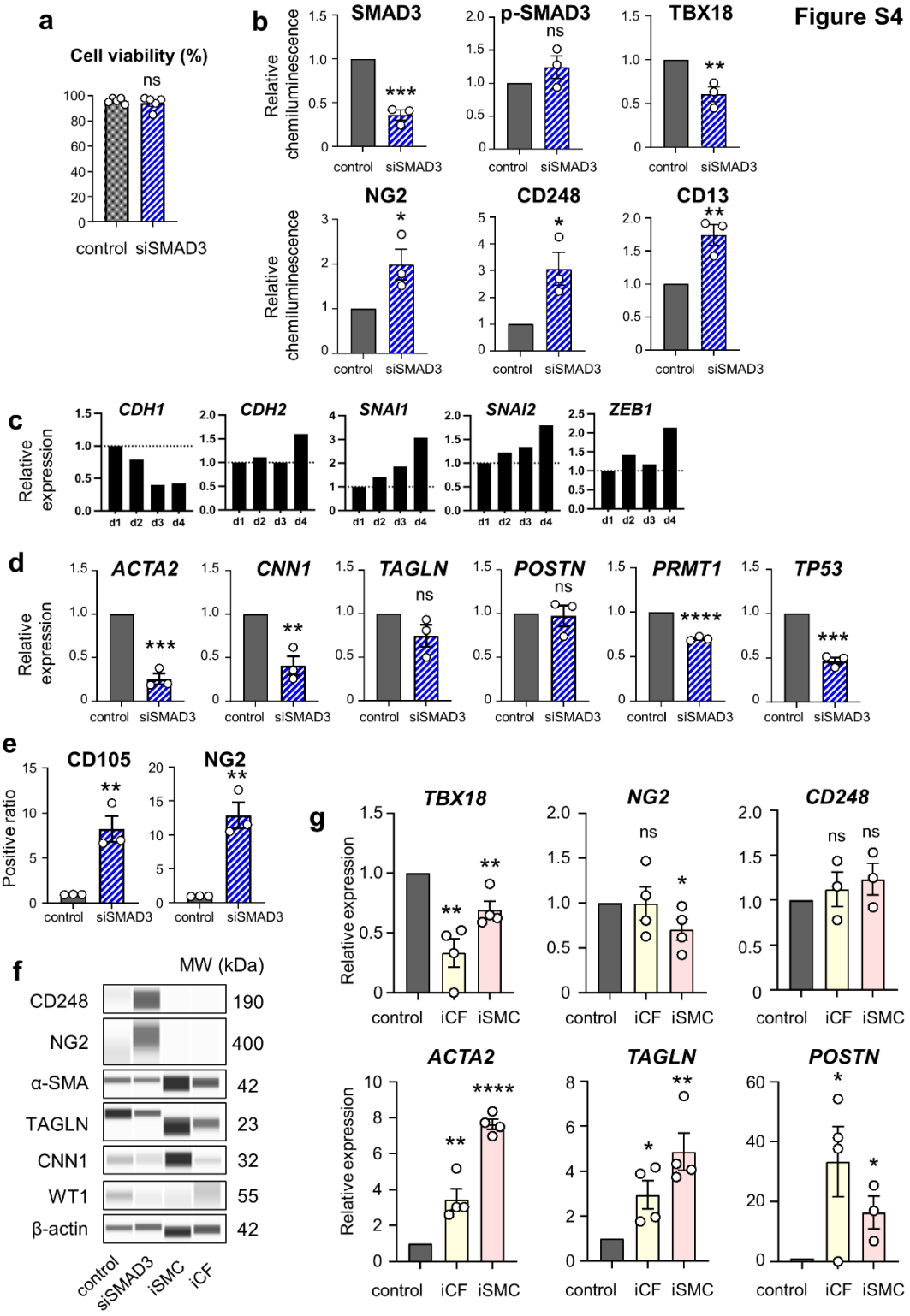
Figure S4
a
Cell viability (%)
ns
control siSMAD3
b
SMAD3
p-SMAD3
TBX18
NG2
CD248
CD13
Relative chemiluminescence
control siSMAD3
c
CDH1
CDH2
SNAI1
SNAI2
ZEB1
Relative expression
d1 d2 d3 d4
d
ACTA2
CNN1
TAGLN
POSTN
PRMT1
TP53
Relative expression
e
CD105
NG2
Positive ratio
f
MW (kDa)
CD248 190
NG2 400
α-SMA 42
TAGLN 23
CNN1 32
WT1 55
β-actin 42
control siSMAD3 iSMC iCF
g
TBX18
NG2
CD248
ACTA2
TAGLN
POSTN
Relative expression
control iCF iSMC

Figure S5

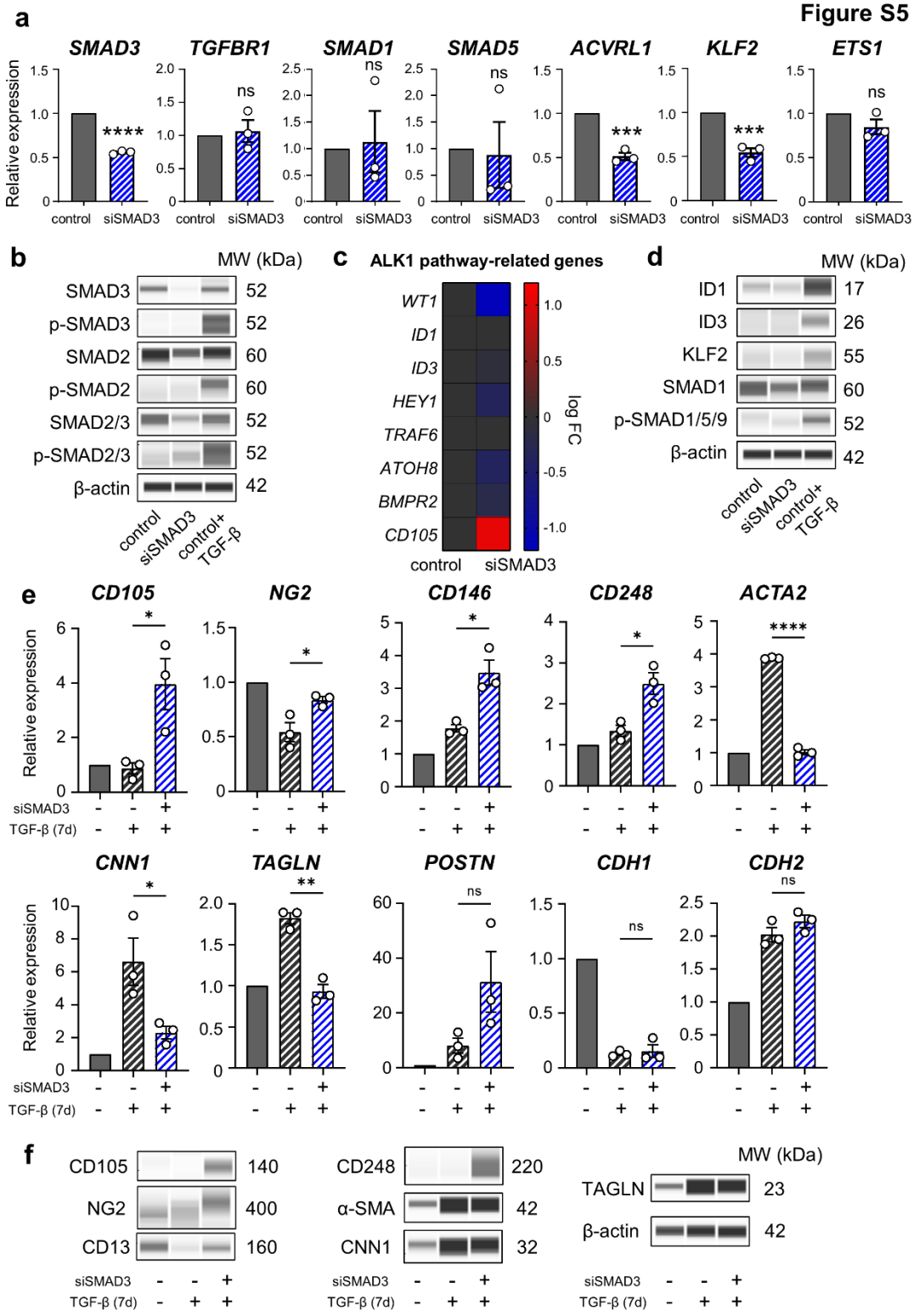
Figure S5
a
SMAD3
TGFBR1
SMAD1
SMAD5
ACVRL1
KLF2
ETS1
Relative expression
control
siSMAD3
b
MW (kDa)
SMAD3
p-SMAD3
SMAD2
p-SMAD2
SMAD2/3
p-SMAD2/3
β-actin
control
siSMAD3
control+ TGF-β
c
ALK1 pathway-related genes
WT1
ID1
ID3
HEY1
TRAF6
ATOH8
BMPR2
CD105
log FC
d
ID1
ID3
KLF2
SMAD1
p-SMAD1/5/9
β-actin
e
CD105
NG2
CD146
CD248
ACTA2
CNN1
TAGLN
POSTN
CDH1
CDH2
siSMAD3
TGF-β (7d)
f
CD105
NG2
CD13
CD248
α-SMA
CNN1
TAGLN
β-actin

Figure S6

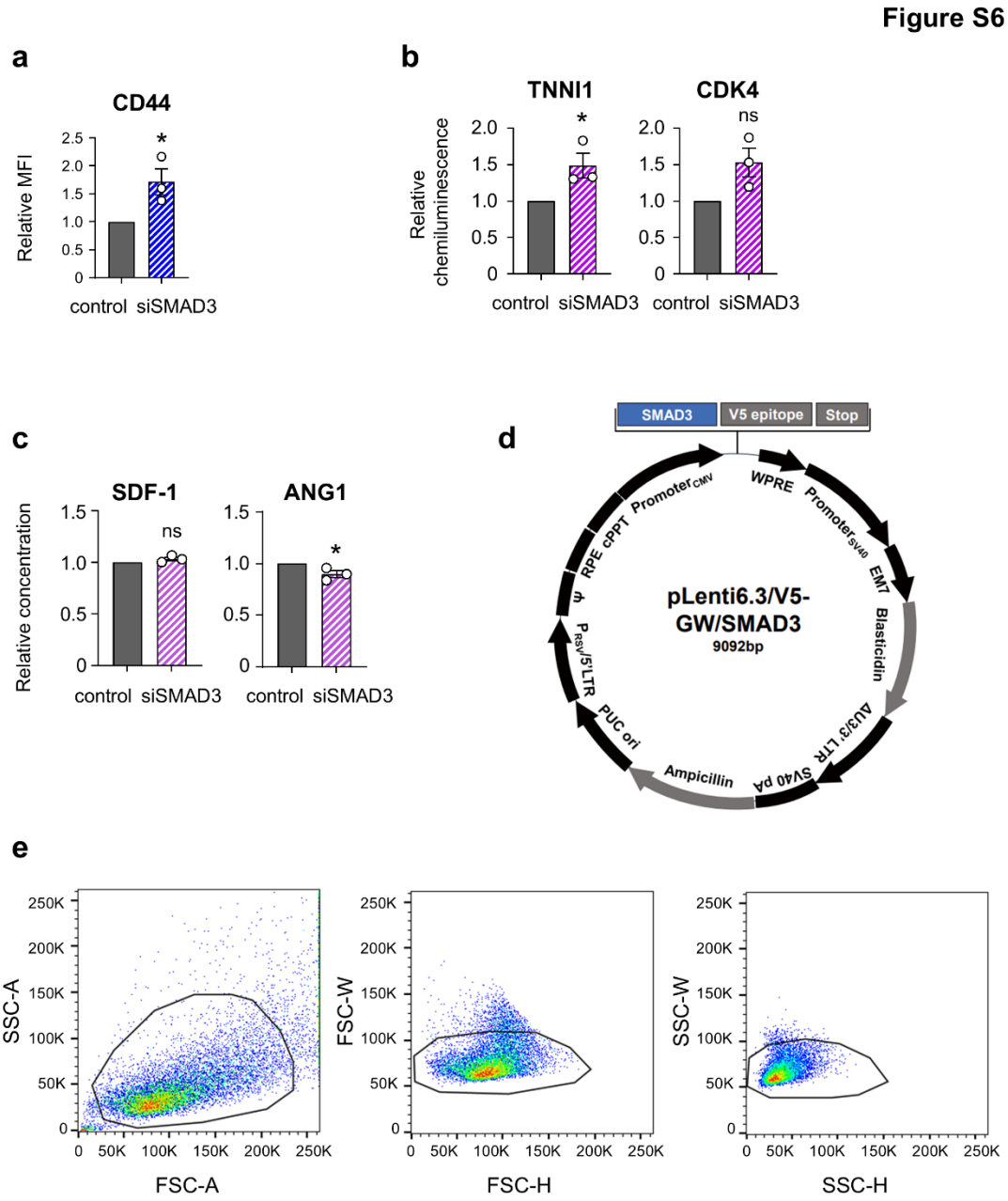
Figure S6
a
CD44
Relative MFI
control siSMAD3
b
TNNI1
CDK4
ns
Relative chemiluminescence
control siSMAD3 control siSMAD3
c
SDF-1
ANG1
ns
Relative concentration
control siSMAD3 control siSMAD3
d
SMAD3
V5 epitope
Stop
pLenti6.3/V5-GW/SMAD3
9092bp
Promoter CMV
WPRE
Promoter SV40
EM7
Blasticidin
ΔU3/3' LTR
SV40 pA
Ampicillin
PUC ori
P RSV/5' LTR
Ψ
RPE cPPT
e
SSC-A
FSC-A
FSC-W
FSC-H
SSC-W
SSC-H

Figure S1. Detailed SMAD3 dynamics and distribution in epicardial development including the effect of SB431542 (related to Figure 1)

(a). The quantitative analysis of Western blot in Figure 1j (TBX18) and 1l (SMAD3 and p-SMAD3) using relative value of the chemiluminescence [n=3; *p = 0.019, ***p = 0.0006, ns = not significant]. **(b,c).** Immunocytochemistry images for total SMAD3 **(b)** and phosphorylated SMAD3 **(c)** in hiPSC-derived epicardial cell (EPI cells) at day 12 and day 24. **(d).** Time-course analysis for proteins of total and phosphorylated SMAD3 at day 12, day 24, day 48, and a positive control (day 24+TGF-β for 1 hour). **(e).** Quantitative RT-PCR (qRT-PCR) analysis of *SMAD3* in hiPSCs and EPI cells at day 24 [n=6; *p = 0.015]. **(f).** qRT-PCR analysis of *SMAD3* and epicardial markers (*WT1* and *TBX18*) in control EPI cells (SB431542+) and EPI cells without SB431542 for 7 days [n=4, ****p < 0.0001, ns = not significant]. **(g).** Western blot analysis of total and phosphorylated SMAD3 in control EPI cells (SB431542+) and EPI cells without SB431542 for 7 days. [All error bars represent standard error of the mean (SEM); the graph plots are derived from experimental replicates, obtained from independent batches; statistical analysis was performed using a two-sided unpaired Student's t-test; scale bars equal 10 μm.]

Figure S2. Information of housekeeping genes and quantification of protein analysis in the context of EMT (related to Figure 2)

(a). CT values in quantitative RT-PCR (qRT-PCR) analysis of housekeeping genes (*GAPDH, ACTB,* and *TBP)* in hiPSC-derived epicardial cells (EPI cells) treated with TGF-β and SB431542 for three days, post day 24 [*p < 0.05, ns = not significant]. **(b).** The quantitative analysis of Western blot in Figure 2f (α-SMA, TAGLN, and CNN1) and 2h (SMAD3 and p-SMAD3) using relative value of the chemiluminescence [n=3 or 4; *p < 0.05, **p < 0.01]. [All error bars represent standard error of the mean (SEM); the graph plots are derived from experimental replicates, obtained from independent batches; statistical analysis was performed using a two-sided unpaired Student's t-test.]

Figure S3. Comparison of endogenous and overexpressed SMAD3 under TGF-β stimulation and overexpression at more immature stage (related to Figure 3)

(a). Immunocytochemistry images for total and phosphorylated SMAD3 in control and SMAD3-overexpressing hiPSC-derived epicardial cell (EPI cells) with or without TGF-β for 1 hour. **(b).** Western blot analysis of total and phosphorylated SMAD3 in control and SMAD3-overexpressing EPI cells with or without TGF-β for 1 hour. **(c).** The quantitative analysis of Western blot in Figure 3d (SMAD3 and p-SMAD3) using relative value of the chemiluminescence [n=3; *p = 0.020, ns = not significant]. **(d).** Quantitative RT-PCR (qRT-PCR) analysis for *SNAI1, SNAI2,* and *ZEB1* [n=4; *p = 0.015]. **(e,f).** Validation of SMAD3 overexpression in qRT-PCR **(e)** and Western blot **(f)** in EPI cells overexpressed SMAD3 from day 12 [n=3; *p = 0.022]. **(g).** qRT-PCR for mature epicardial markers: *UPK3B, MSLN, ITLN1, EFEMP1,* and *C3* [n=4: *p < 0.05, ns = not significant]. [All error bars represent standard error of the mean (SEM); the graph plots are derived from experimental replicates, obtained from independent batches; statistical analysis was performed using a two-sided unpaired Student's t-test; scale bars equal 10 μm.]

Figure S4. Characterization of specific EMT towards cardiac pericyte progenitors induced by SMAD3 silencing (related to Figure 4)

(a). Cell viability of SMAD3-silenced and control hiPSC-derived epicardial cell (EPI cells) 7 days after siRNA transfection [n=5; ns = not significant]. **(b).** The quantitative analysis of Western blot in Figure 4d (SMAD3 and p-SMAD3), 4h (TBX18), and 4i (NG2, CD248, and CD13) using relative value of the chemiluminescence [n=3; *p < 0.05, **p < 0.01, ***p = 0.0004]. **(c).** Time-course (days) of the mRNA expression analysis (RNA-Seq derived) for *CDH1, CDH2, SNAI1, SNAI2* and *ZEB1* during SMAD3 downregulation in EPI cells. Values are expressed as a ratio to day 1. **(d).** Quantitative RT-PCR (qRT-PCR) analysis of SMAD3-silenced EPI cells (siSMAD3) compared to control for the EPDC markers (*ACTA2, CNN1, TAGLN* and *POSTN*) and *PRMT1* and *TP53* [n=3; ** p < 0.01, ***p < 0.001, ****p < 0.0001, ns = not significant]. **(e).** The quantitative analysis of flow cytometry in Figure 4l-o, indicating the

percentage of positive population for CD105 and NG2 [n=3, **p < 0.01]. **(f).** Protein expression analysis for cardiac pericytes, EPDCs and epicardial markers (cardiac pericytes- NG2, CD248; EPDCs- α-SMA, TAGLN, and CNN1; epicardial- WT1) in control and siSMAD3-EPI cells compared to induced cardiac fibroblasts (iCF) and induced smooth muscle cells (iSMC). **(g).** qRT-PCR analysis for epicardial, cardiac pericytes and EPDCs markers (epicardial- *TBX18*; cardiac pericytes- *NG2, CD248*; EPDCs- *ACTA2, TAGLN,* and *POSTN*) in iCF and iSMC compared to control EPI cells maintained in SB431542 [n=3 or 4; *p < 0.05, ** p < 0.01, ****p < 0.0001, ns = not significant].

[All error bars represent standard error of the mean (SEM); the graph plots are derived from experimental replicates, obtained from independent batches; statistical analysis was performed using a two-sided unpaired Student's t-test; scrambled siRNA was used as a control.]

Figure S5. Dynamics of ALK5 and ALK1 pathway-related markers and the independent role of SMAD3 silencing from canonical TGF-β pathway (related to Figure 4)

(a). Quantitative RT-PCR (qRT-PCR) analysis of SMAD3-silenced (siSMAD3) hiPSC-derived epicardial cell (EPI cells) compared to control for ALK5-related genes (*SMAD3* and *TGFBR1*) and ALK1-related genes (*SMAD1, SMAD5, ACVRL1, KLF2* and *ETS1*) [n=3; ***p < 0.001, ****p < 0.0001, ns = not significant]. **(b).** Protein expression analysis for ALK5 pathway-related SMAD families, including positive control (treated by TGF-β for 1 hour). **(c).** RNA-seq-derived heatmap illustrating ALK1 pathway-related genes and transcriptional factors, including WT1 and CD105 as a negative and positive control. **(d).** Protein expression analysis for ALK1 pathway-related SMAD families and the downstream markers with positive control. **(e).** qRT-PCR analysis for cardiac pericyte markers (*CD105, NG2, CD146,* and *CD248*), EPDCs markers (*ACTA2, CNN1, TAGLN,* and *POSTN*), *CDH1* and *CDH2* of TGF-β-treated cells with scrambled siRNA (siSMAD3-) or siSMAD3 (siSMAD3+) compared to control EPI cells. **(f).** Protein expression analysis for cardiac pericyte markers (CD105, NG2, CD13, and CD248)

and EPDCs markers (α-SMA, CNN1, and TAGLN) of TGF-β-treated cells with scrambled siRNA (siSMAD3-) or siSMAD3 (siSMAD3+) compared to control EPI cells.

[All error bars represent standard error of the mean (SEM); the graph plots are derived from experimental replicates, obtained from independent batches; statistical analysis was performed using a two-sided unpaired Student's t-test; scrambled siRNA was used as a control.]

Figure S6. Information of protein analysis quantification and other data in relation to each experiment (related to Figures 5, 6 and experimental procedures)

(a). The quantitative analysis of flow cytometry in Figure 5e, indicating the relative value of mean fluorescence intensity (MFI) for CD44 [n=3, *p = 0.038]. **(b).** The quantitative analysis of Western blot in Figure 6g (TNNI1 and CDK4) using relative value of the chemiluminescence [n=3; *p = 0.047, ns = not significant]. **(c).** ELISA showing that SDF-1 and ANG1 concentration in the supernatant from SMAD3-silenced EPI cells was 73.3 and 154.2 pg/ml, 1.03 and 0.90 times compared to control, respectively [n=3; *p = 0.041, ns = not significant]. **(d).** Schematic representation of pLenti6.3/V5-GW/SMAD3 containing human SMAD3 cDNA under a CMV promoter for gene overexpression. **(e).** Representative FSC/SSC gating and doublet elimination using FSC and SSC.

[All error bars represent standard error of the mean (SEM); the graph plots are derived from experimental replicates, obtained from independent batches; statistical analysis was performed using a two-sided unpaired Student's t-test; scrambled siRNA was used as a control.]

Table S1: List of qRT-PCR primers (related to figures 1, 2, 3, 4, 6, S1, S2, S3, S4, S5)

Genes	**Sequences (5'-3')**
GAPDH	**F:** GGAGCGAGATCCCTCCAAAAT
	R: GGCTGTTGTCATACTTCTCATGG
WT1	**F:** CAGCTTGAATGCATGACCTG
	R: GATGCCGACCGTACAAGAGT
TBX18	**F:** TTAACCTTGTCCGTCTGCCTGAGT
	R: GTAATGGGCTTTGGCCTTTGCACT
SMAD2	**F:** CGTCCATCTTGCCATTCACG
	R: CTCAAGCTCATCTAATCGTCCTG
SMAD3	**F:** TGGACGCAGGTTCTCCAAAC
	R: CCGGCTCGCAGTAGGTAAC
SMAD4	**F:** CTCATGTGATCTATGCCCGTC
	R: AGGTGATACAACTCGTTCGTAGT
CDH1	**F:** CGAGAGCTACACGTTCACGG
	R: GGGTGTCGAGGGAAAAATAGG
CDH2	**F:** CTCCAATCAACTTGCCAGAA
	R: ATACCAGTTGGAGGCTGGTC
ACTA2	**F:** TCAATGTCCCAGCCATGTAT
	R: CAGCACGATGCCAGTTGT
CNN1	**F:** CTGTCAGCCGAGGTTAAGAAC
	R: GAGGCCGTCCATGAAGTTGTT
PDGFRA	**F:** TGGCAGTACCCCATGTCTGAA
	R: CCAAGACCGTCACAAAAAGGC
TAGLN	**F:** AGTGCAGTCCAAAATCGAGAAG
	R: CTTGCTCAGAATCACGCCAT
ALDH1A2	**F:** CTCCTCTGTCACACCCCATT
	R: TTGACAGCTGGAAAGATGGA
BNC1	**F:** CTGTACTCTGAACTGTAGTTGCC
	R: CCATGCTTGCATTGGTCACAC
UPK3B	**F:** TGCCCTACACACCACAGATAA
	R: GCAAGCCCATCGAAGACAC
MSLN	**F:** CCAACCCACCTAACATTTCCAG
	R: CAGCAGGTCCAATGGGAGG
ITLN1	**F:** ACGTGCCCAATAAGTCCCC
	R: CCGTTGTCAGTCCAACACTTTC
EFEMP1	**F:** GTCACAGGACACCGAAGAAAC
	R: TTGCATTGCTGTCTCACAGGA
C3	**F:** GGGGAGTCCCATGTACTCTATC
	R: GGAAGTCGTGGACAGTAACAG
RGS5	**F:** GACATGGCCCAGAAAAGAATCC
	R: CACAAAGCGAGGCAGAGAATC
DLK-1	**F:** CTTTCGGCCACAGCACCTAT
	R: TGTCATCCTCGCAGAATCCAT
ANPEP (CD13)	**F:** GACCAAAGTAAAGCGTGGAATCG
	R: TCTCAGCGTCACCCGGTAG
MCAM (CD146)	**F:** AGCTCCGCGTCTACAAAGC
	R: CTACACAGGTAGCGACCTCC
TEM1 (CD248)	**F:** AGTGTTATTGTAGCGAGGGACA
	R: CCTCTGGGAAGCTCGGTCTA
ENG (CD105)	**F:** TGCACTTGGCCTACAATTCCA
	R: AGCTGCCCACTCAAGGATCT

NG2	**F:** CTTTGACCCTGACTATGTTGGC
	R: TGCAGGCGTCCAGAGTAGA
TNNI1	**F:** CCGGAAGTCGAGAGAAAACCC
	R: TCAATGTCGTATCGCTCCTCA
CDK4	**F:** ATGGCTACCTCTCGATATGAGC
	R: CATTGGGGACTCTCACACTCT
CDK6	**F:** TCTTCATTCACACCGAGTAGTGC
	R: TGAGGTTAGAGCCATCTGGAAA
CCND1	**F:** GCTGCGAAGTGGAAACCATC
	R: CCTCCTTCTGCACACATTTGAA
ACTB	**F:** CATGTACGTTGCTATCCAGGC
	R: CTCCTTAATGTCACGCACGAT
TBP	**F:** CCACTCACAGACTCTCACAAC
	R: CTGCGGTACAATCCCAGAACT
SNAI1	**F:** TCGGAAGCCTAACTACAGCGA
	R: AGATGAGCATTGGCAGCGAG
SNAI2	**F:** CGAACTGGACACACATACAGTG
	R: CTGAGGATCTCTGGTTGTGGT
ZEB1	**F:** GATGATGAATGCGAGTCAGATGC
	R: ACAGCAGTGTCTTGTTGTTGT
TP53	**F:** CAGCACATGACGGAGGTTGT
	R: TCATCCAAATACTCCACACGC
PRMT1	**F:** CTTTGACTCCTACGCACACTT
	R: GTGCCGGTTATGAAACATGGA
TGFBR1	**F:** ACGGCGTTACAGTGTTTCTG
	R: GCACATACAAACGGCCTATCTC
SMAD1	**F:** AGAGACTTCTTGGGTGGAAACA
	R: ATGGTGACACAGTTACTCGGT
SMAD5	**F:** CCAGCAGTAAAGCGATTGTTGG
	R: GGGGTAAGCCTTTTCTGTGAG
ACVRL1	**F:** CATCGCCTCAGACATGACCTC
	R: GTTTGCCCTGTGTACCGAAGA
KLF2	**F:** TTCGGTCTCTTCGACGACG
	R: TGCGAACTCTTGGTGTAGGTC
ETS1	**F:** GATAGTTGTGATCGCCTCACC
	R: GTCCTCTGAGTCGAAGCTGTC

Table S2: List of antibodies used in immunocytochemistry (related to figures 1, 4, S1, S3)

Primary antibody	**Source**	**Identifier**	**Host**	**Concentration**
Anti-WT1	Abcam	ab89901	Rabbit	1:50
Anti-TBX18	Abcam	ab115262	Rabbit	1:50
Anti-ZO-1	Invitrogen	ZO1-1A12	Mouse	1:200
Anti-CD105/Endoglin (D50G1)	CST	#4335	Rabbit	1:200
Anti-NG2/CSPG4	CST	#52635	Rabbit	1:200
Anti-SMAD3	Abcam	ab40854	Rabbit	1:500
Anti-phospho-SMAD3	Abcam	ab52903	Rabbit	1:100
Secondary antibody				
Anti-rabbit IgG-Alexa Fluor 647	Life Technologies	A21245	Goat	1:1000
Anti-mouse IgG-Alexa Fluor 488	Life Technologies	A11001	Goat	1:1000

Table S3: List of antibodies used in flow cytometry (related to figures 1, 3, 4, 5, 6, S5, S6)

Primary antibody	**Source**	**Identifier**	**Host**	**Concentration**
Anti-WT1	Abcam	ab89901	Rabbit	1:100
Anti-TBX18	Abcam	ab115262	Rabbit	1:100
Anti-cTnT	Thermo Scientific	MS295P	Mouse	1:100
Secondary antibody				
Anti-rabbit IgG-Alexa Fluor 647	Life Technologies	A21245	Goat	1:1000
Anti-mouse IgG-Alexa Fluor 488	Life Technologies	A11001	Goat	1:1000
Conjugated antibody				
CD105-APC	BD	562408	Mouse	1:100
NG2-Alexa Fluor 647	BD	562414	Mouse	1:100
CD44-PE	BD	550989	Mouse	1:100

Table S4: List of antibodies used in western blotting (Simple Western) (related to figures 1, 2, 3, 4, 6, S1, S3, S4, S5)

Primary antibody	**Source**	**Identifier**	**Host**	**Concentration**
Anti-β-actin	Sigma-Aldrich	A5441	Mouse	1:100
Anti-WT1	Abcam	ab89901	Rabbit	1:20
Anti-TBX18	Abcam	ab115262	Rabbit	1:20
Anti-SMAD3	Abcam	ab40854	Rabbit	1:20
Anti-phospho-SMAD3	Abcam	ab52903	Rabbit	1:20
Anti-α-SMA	Abcam	ab7817	Mouse	1:20
Anti-TAGLN	CST	#40471	Rabbit	1:20
Anti-CNN1	CST	#17819	Rabbit	1:20
Anti-CD105/Endoglin (D50G1)	CST	#4335	Rabbit	1:20
Anti-NG2/CSPG4	CST	#52635	Rabbit	1:20
Anti-CD248	CST	#47948	Rabbit	1:20
Anti-CD13	Abcam	ab108310	Rabbit	1:20
Anti-CDK4	CST	12790	Rabbit	1:20
Anti-CDK6	CST	13331	Rabbit	1:20
Anti-TNNI1	Abcam	203515	Rabbit	1:20
Anti-SMAD2	CST	#5339	Rabbit	1:20
Anti-phospho-SMAD2	CST	#3108	Rabbit	1:20
Anti-SMAD2/3	CST	#3102	Rabbit	1:20
Anti-phospho-SMAD2/3	CST	#8828	Rabbit	1:20
Anti-ID1	CST	#23369	Rabbit	1:20
Anti-ID3	CST	#9837	Rabbit	1:20
Anti-KLF2	CST	#15306	Rabbit	1:20
Anti-SMAD1	CST	#6944	Rabbit	1:20
Anti-phospho-SMAD1/5/9	CST	#13820	Rabbit	1:20
Secondary antibody				
Anti-Rabbit Secondary HRP Antibody	Bio-Techne	042-206	Goat	1:1
Anti-Mouse Secondary HRP Antibody	Bio-Techne	042-205	Goat	1:1

Table S5: List of ELISA kits (related to figures 6, S6)

Name	Source	Identifier
Human VEGFA ELISA Kit	Abcam	ab119566
Human Angiopoietin 1 ELISA Kit (ANG1)	Abcam	ab99972
Human SDF1 alpha ELISA Kit	Abcam	ab100637

Supplemental experimental procedures

Epicardial differentiation

For epicardial differentiation, on day 0, iPSCs were dissociated using Accumax and embryoid bodies (EBs) were generated in HEMA-coated plates (6000 cells/well, for 96-well plate). We used 70 µl/well of the following differentiation media on day 0: StemPro®-34 media supplemented with 2 mM L-glutamine, 50 µg/ml ascorbic acid (AA), 0.4 µM monothioglycerol (MTG), 150 µg/ml transferrin, 10 µM Y-27632, 2 ng/ml rh BMP4 and 0.5% Matrigel. After 24 hours, we added more 70 µl/well media to obtain the following final concentration: 2 mM L-glutamine, 50 µg/ml AA, 0.4 µM MTG, 150 µg/ml transferrin, 10 µM Y-27632, 10 ng/ml rh BMP4, 2 ng/ml Activin A and 5 ng/ml rh bFGF. On day 3.5, EBs were collected and dissociated using Accumax, subsequently plated onto 0.1% gelatin-coated dishes (0.25×10^5 cells/cm^2), suspended in the following differentiation media: StemPro®-34 media supplemented with 2 mM L-glutamine, 50 µg/ml AA, 0.4 µM MTG, 150 µg/ml transferrin, 10 µM Y-27632, 3 mM CHIR99021, 30 ng/ml rh BMP4, 5 ng/ml rh VEGF and 10 µM SB431542. On day 6, the media was changed with the same media on day 3.5 only without Y-27632. On day 8, hiPSC-derived epicardial cells were maintained in the following maintenance media: DMEM high glucose containing 10% FBS and 10 µM SB431542. EPI cells were detached by Accumax, dissociated, and replated as described above not to be too confluent, approximately every 5 days.

Cardiomyocyte differentiation

For cardiomyocytes differentiation, the protocol is same for epicardial differentiation on day 0, except that the cell number is 2 million/well for 6-well plate and the amount of the media is 1.5 ml/well. On day 1, we added more 1.5ml/well media to get the following final concentration (induction media 1): 2 mM L-glutamine, 50 µg/ml AA, 0.4 µM MTG, 150 µg/ml transferrin, 10 µM Y-27632, 10 ng/ml rh BMP4, 6 ng/ml Activin A and 5 ng/ml rh bFGF. On day 3, EBs were washed by IMDM and suspended in the following media (induction media 2): StemPro®-34 media supplemented with 2 mM L-glutamine, 50 µg/ml AA, 0.4 µM MTG, 150 µg/ml transferrin, 10 ng/ml rh VEGF and 1 µM IWP-3. On day 7, the media were changed to the following media

(induction media 3): StemPro®-34 media supplemented with 2 mM L-glutamine, 50 µg/ml AA, 0.4 µM MTG, 150 µg/ml transferrin and 5 ng/ml rh VEGF. After that, media change was performed with the induction media 3 every 2-3 days until day 16. On day 16, cells were detached by collagenase I and Accumax and plated onto fibronectin-coated dishes (1.0×10^5 cells/cm^2), suspended in the induction media 3. After that, media change with the induction media 3 was performed every 2-3 days until day 23.

Cardiac fibroblasts and Smooth muscle cells differentiation (EMT induction)

For differentiation towards CF, EPI cells at day 24 were treated with 10 ng/ml bFGF for 8 days. For differentiation towards SMC, EPI cells at day 24 were treated with 5 ng/ml TGF-β for 4 days followed by 10 ng/ml bFGF for another 4 days.

Endothelial Tube Formation assay (*in vitro* Angiogenesis)

For the endothelial tube formation assay, we first added 35 µl of Matrigel to each well of a pre-chilled 96-well plate and incubated it for 1 hour at 37ºC. After incubation, a cell suspension (totaling 3.0×10^4 cells per 150 µl) was added to each well and then incubated at 37ºC. In co-culture experiments, we utilized Human Aortic Endothelial Cells (HAEC) along with only the EGFP-positive population in EGFP-overexpressed iPSC-derived epicardial cells (EPI cells). The cell suspension was prepared by mixing them in a 1:1 ratio. Images were acquired 4 to 12 hours after starting the assay. Tube formation was quantified by counting the number of junctions in a rectangle measuring 3623.44 µm in length and 2717.58 µm in width using ImageJ.

Assessment of paracrine signaling by SMAD3-downregulating cells on the proliferative activity of hiPSC-derived cardiomyocytes.

FUCCI-expressing hiPSCs were differentiated into cardiomyocytes as described previously. On day 23, the cardiomyocytes were cultured for 36 hours in supernatants from either SMAD3-silenced or control EPI cells. These supernatants were collected 7 days post-siRNA transfection. In this experiment, the media was changed only once, 24 hours after siRNA transfection, and SB431542 was added to the media every few days thereafter.

Image acquisition, processing, and analysis

Microscopy images were taken by BZ-X710 and BZ-X 810 (Keyence). A representative section was chosen, cropped and magnified for phase contrast and fluorescence pictures. For counting the number of junctions in endothelial tube formation assay, navigation mode was used. General image analysis was performed using Microsoft power point as well as ImageJ v1.54d.

Real-time quantitative PCR analysis

Live cells were collected in QIAzol lysis reagent. Total RNA was purified using the RNAeasy Micro Kit (Qiagen) according to the provided protocol. Complementary DNA was synthesized using the ReverTra Ace system (Toyobo BIOTECH) according to the manufacturer's manual. Quantitative RT-PCR was performed using THUNDERBIRD™ Next SYBR® qPCR mix with primers (summarized in Table S1). Data acquisition was carried out by QuantStudio™ 3 Real-Time PCR System (Applied Biosystems™). All data were acquired from at least three biological replicates and analyzed using Microsoft Excel and GraphPad Prism v9. The relative mRNA expression was normalized to GAPDH and calculated using the relative fold change ($2^{-\Delta\Delta CT}$) in each gene.

We excluded values that lacked any recorded data. When dealing with datasets that displayed a normal distribution, we performed a Student's t-test, as specified in the figure legends. All error bars in our graphs represent the standard error of the mean (SEM).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 30 min at room temperature and stored in PBS at 4ºC. Blocking was performed for 30 min in blocking buffer: 1% BSA, 0.5% Triton X and 0.1M glycine in PBS. Following PBS washing, primary antibody was added in staining buffer (blocking buffer without glycine) and incubated overnight at 4ºC. After incubation and PBS washing, secondary antibody was added in staining buffer and incubated for 2 hours at 4ºC. After incubation and PBS washing, Hoechst (Life Technologies; H3570, 1:10000) was added for nuclear counterstaining. The information of used antibodies and concentrations were shown in Table S2.

Flow cytometry analysis/FACS

Cells were dissociated by Accumax, washed twice, and fixed with 4% paraformaldehyde for 30 min at room temperature. In case for nuclear staining, following PBS washing, the cells were permeabilized by 90% methanol for 30min at 4 ºC. After permeabilization and PBS washing, primary antibody was diluted in FACS Buffer (10% FBS in PBS) and incubated overnight at 4ºC. The next day, following PBS washing, secondary antibody was added in FACS Buffer and incubated for 2 hours at 4ºC. In case for cell surface marker staining, following PBS washing, conjugated antibody was diluted in FACS buffer and incubated at room temperature for 30 min. Stained cells were analyzed and sorted using FACSAria II (BD Biosciences). Data were analyzed using FlowJo v10.6.1 analysis software (BD Biosciences). The information of used antibodies and concentrations were shown in Table S3.

We identified the cell population by using FSC/SSC gating, and we distinguished doublets based on the method explained in Figure S6e. To establish the negative gates, we utilized unstained samples or isotype controls, and we also included negative control samples in our analysis.

Western blotting (Simple Western)

Cells were detached and collected in Mammalian Protein Extraction Reagent (M-PER) (Thermo Scientific; 78501). The amount of protein was determined by using Quick Start™ Bradford Protein Assay (Bio-Rad) and EnVision 2104 plate reader (Perkin Elmer) as the standard. We used a Wes™ instrument (ProteinSimple) as protocol instructed. The information of used antibodies and concentrations were shown in Table S4. The chemiluminescence of protein was quantified using Compass for SW software v6.2.0 (ProteinSimple), although WT1, CD105, and CDK6 were difficult to quantify because of the problems with sensitivity of the antibody.

Enzyme-linked immunosorbent assay (ELISA)

The levels of VEGFA, SDF-1, and ANG1 were assessed using ELISA kits shown in Table S5, following the manufacturer's manual. Absorbance at 450 nm was measured using EnVision 2104 plate reader (Perkin Elmer).