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Supplemental Information

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Adhesion GPCR, latrophilin-2, specifies cardiac lineage commitment through CDK5, Src, and P38MAPK

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Figure S1

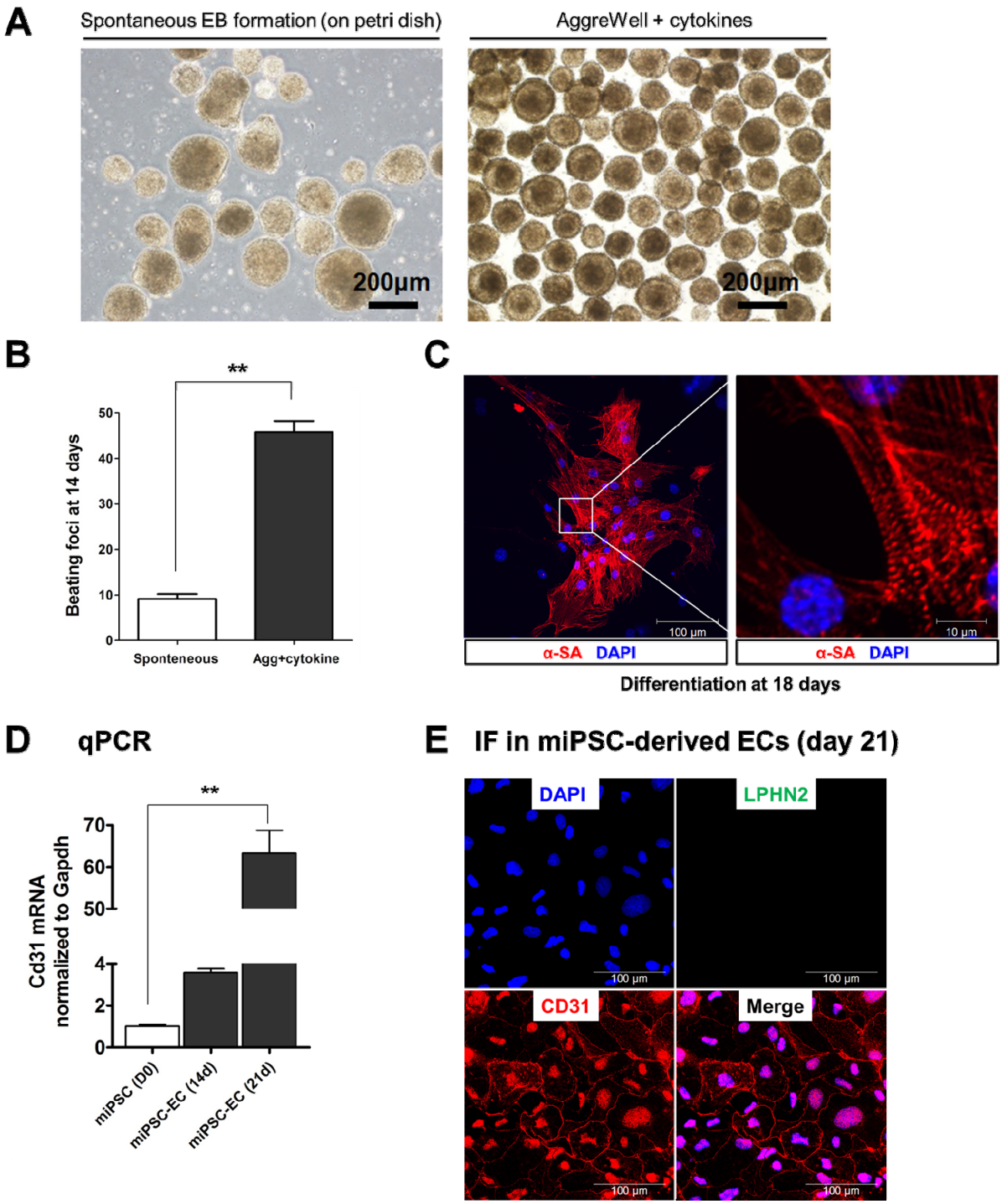

Figure S1 (Related to Figure 1). Optimized culture conditions for cardiac differentiation and LPHN2 expression in mouse iPSC-derived ECs.

(**A**) Comparison of the size and shape of EBs using Petri dishes and AggreWell plates at day 4 after differentiation. The EB populations on AggreWell plates are uniform in size and shape relative to those of the petri dish culture. Scale bar, 200 μm. **(B)** Beating foci count in cardiac differentiation culture at 14 days. $^{**}P < 0.01$, Mann–Whitney U test, $n = 8$ independent biological replicates. **(C)** Immunostaining for α-sarcomeric actinin (α-SA, red) in cardiac differentiation culture at day 18. Blue, nuclear counterstain with 4, 6-diamidino-2-phenylindole (DAPI). A white rectangle in the left image indicates the regions shown at higher magnification. Scale bars, 100 μm (left panels) or 10 μm (right panels). **(D)** Gene expression analysis of mouse iPSC-derived endothelial cells analyzed by qPCR for *Cd31*. Expression values are shown relative to that of miPSCs (D0). $^{**}P < 0.01$, ANOVA test and *post hoc* Bonferroni test, $n = 3$ independent replicates. **(E)** Immunostaining for LPHN2 (green) and CD31 (red) in mouse iPSC-derived ECs. Scale bar, 100 μm. Scale bar, 100 μm.

Figure S2

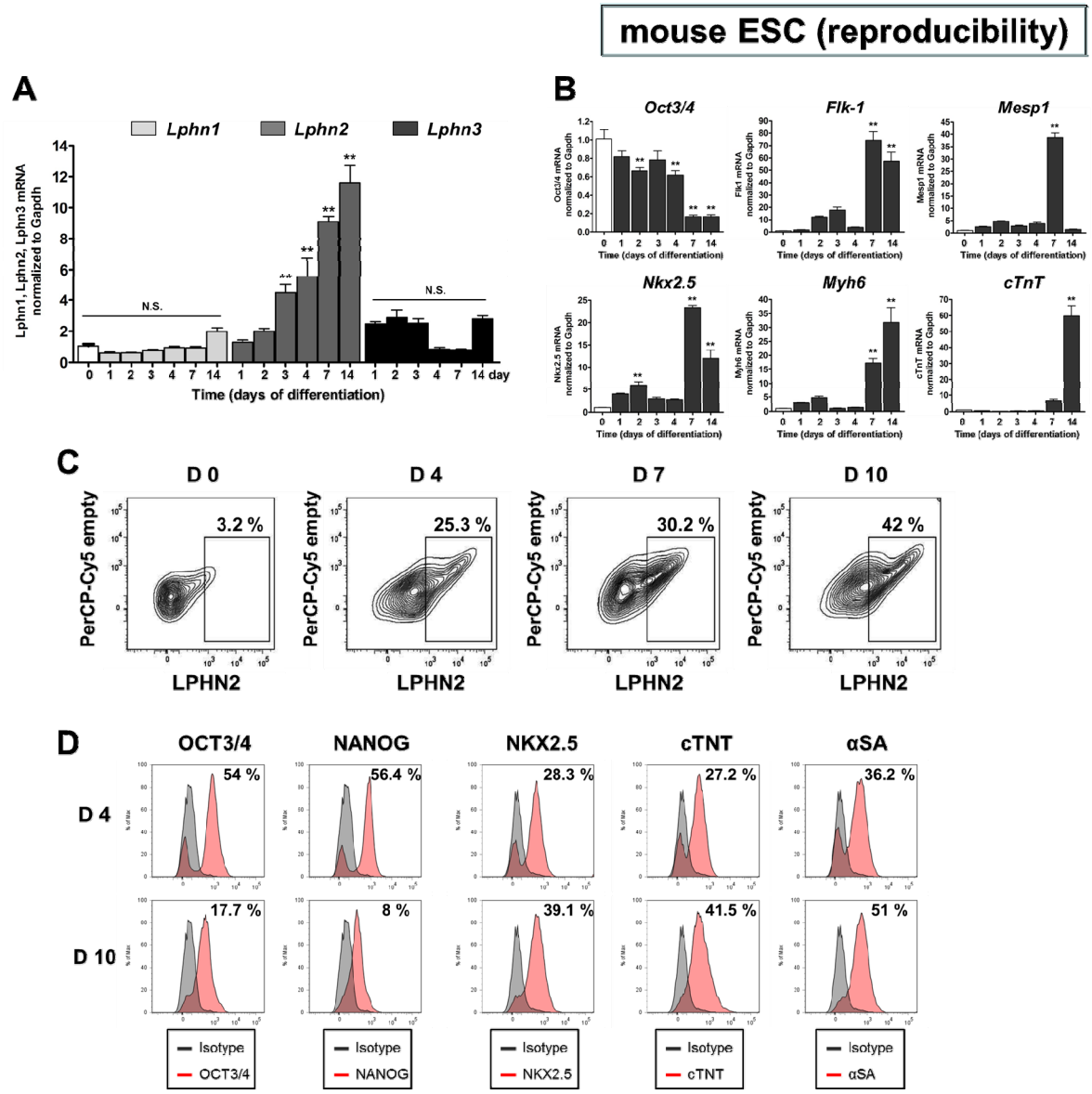

Figure S2 (Related to Figure 1). Reproducibility of iPSC findings in mouse ESCs.
(A) Gene expression analysis by qPCR of *Lphn1*, *Lphn2,* and *Lphn3* in ESCs following cardiac differentiation. Values are shown relative to day 0. $^{**}P < 0.01$, N.S. (not significant), ANOVA test and *post hoc* Bonferroni test, $n = 3$ independent replicates. **(B)** mRNA expression levels of *Oct3/4*, *Flk-1*, *Mesp1*, *Nkx2.5*, *Myh6,* and *cTnT* in ESC-derived cells during cardiac differentiation. $^{**}P < 0.01$, ANOVA test and *post hoc* Bonferroni test, $n = 3$ independent replicates. **(C)** Sequential changes in LPHN2 expression in ESC-derived cardiac lineage populations by FACS analysis. **(D)** FACS analysis of multiple markers (OCT3/4, NANOG, NKX2.5, cTNT, and αSA) in ESC-derived cells during cardiac differentiation.

Figure S3

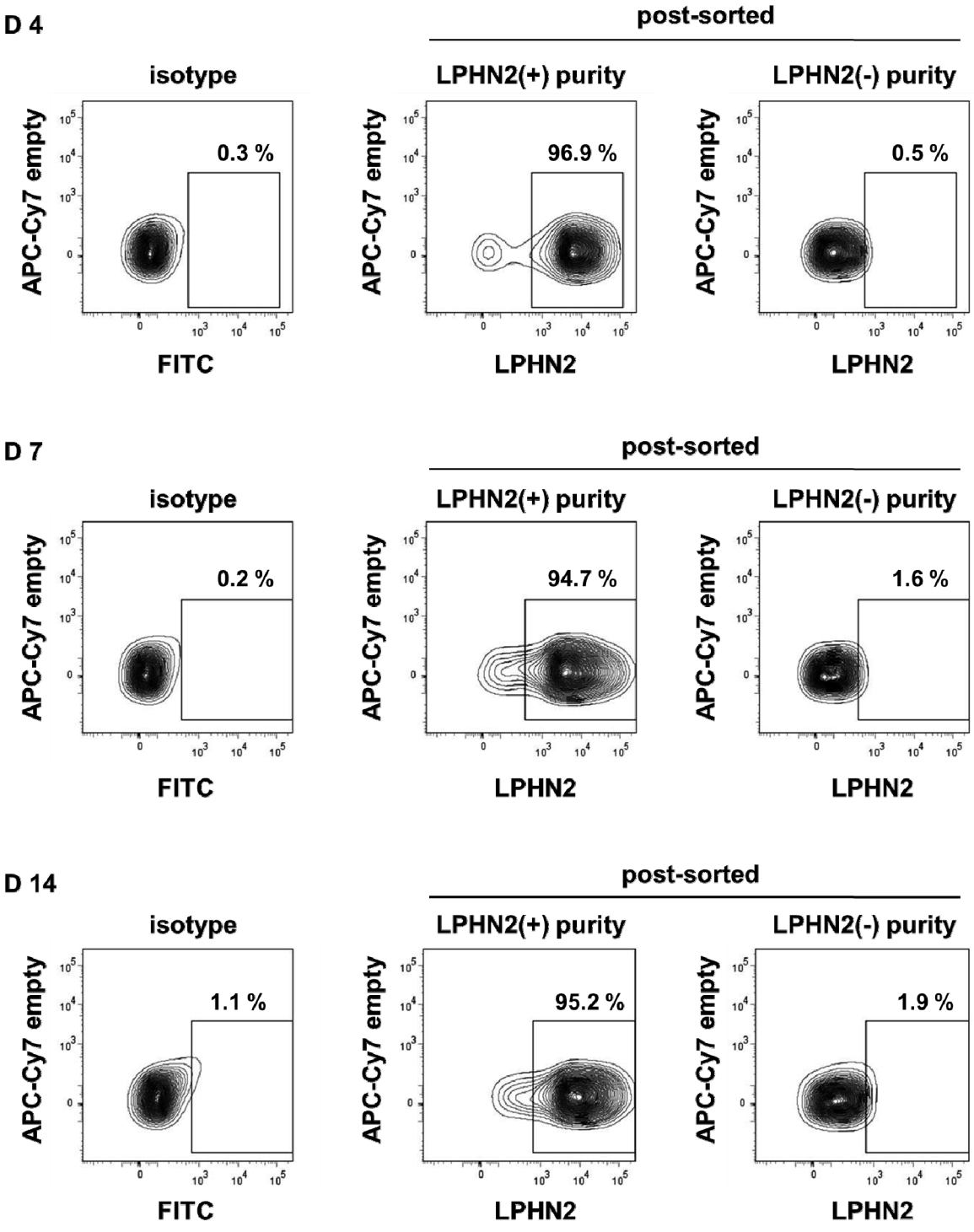

Figure S3 (Related to Figure 2). Sorting purity of LPHN2^{+} and LPHN2^{-} fractions in iPSC-derived cells during cardiac differentiation.

Figure S4

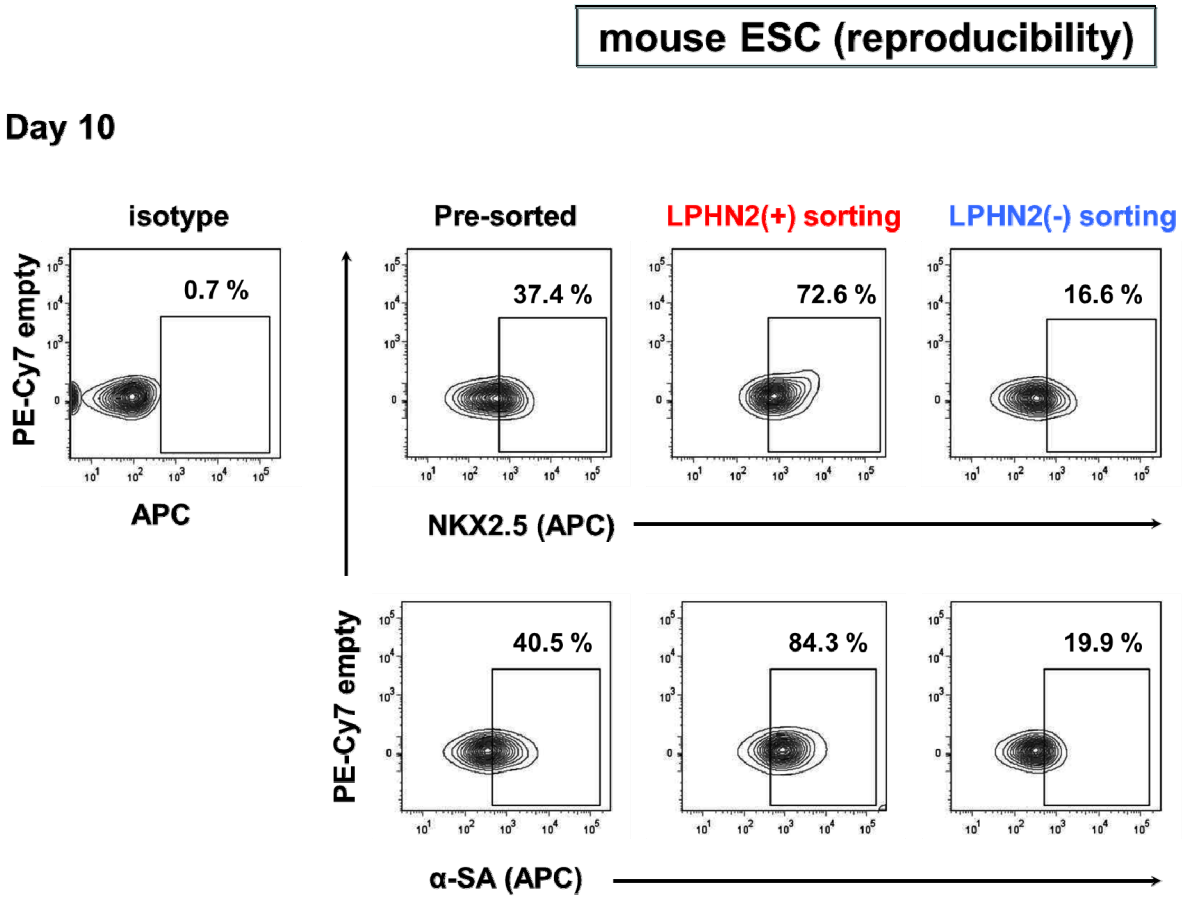

Figure S4 (Related to Figure 2). Enriched expression of NKX2.5 and α-SA sorted by LPHN2 in mouse ESCs.

Representative FACS plot showing surface expression of NKX2.5 and α-SA at 10 days after cardiac differentiation for pre-sort, $LPHN2^{+}$, and $LPHN2^{-}$ fraction in mouse ESC-derived cells.

Figure S5

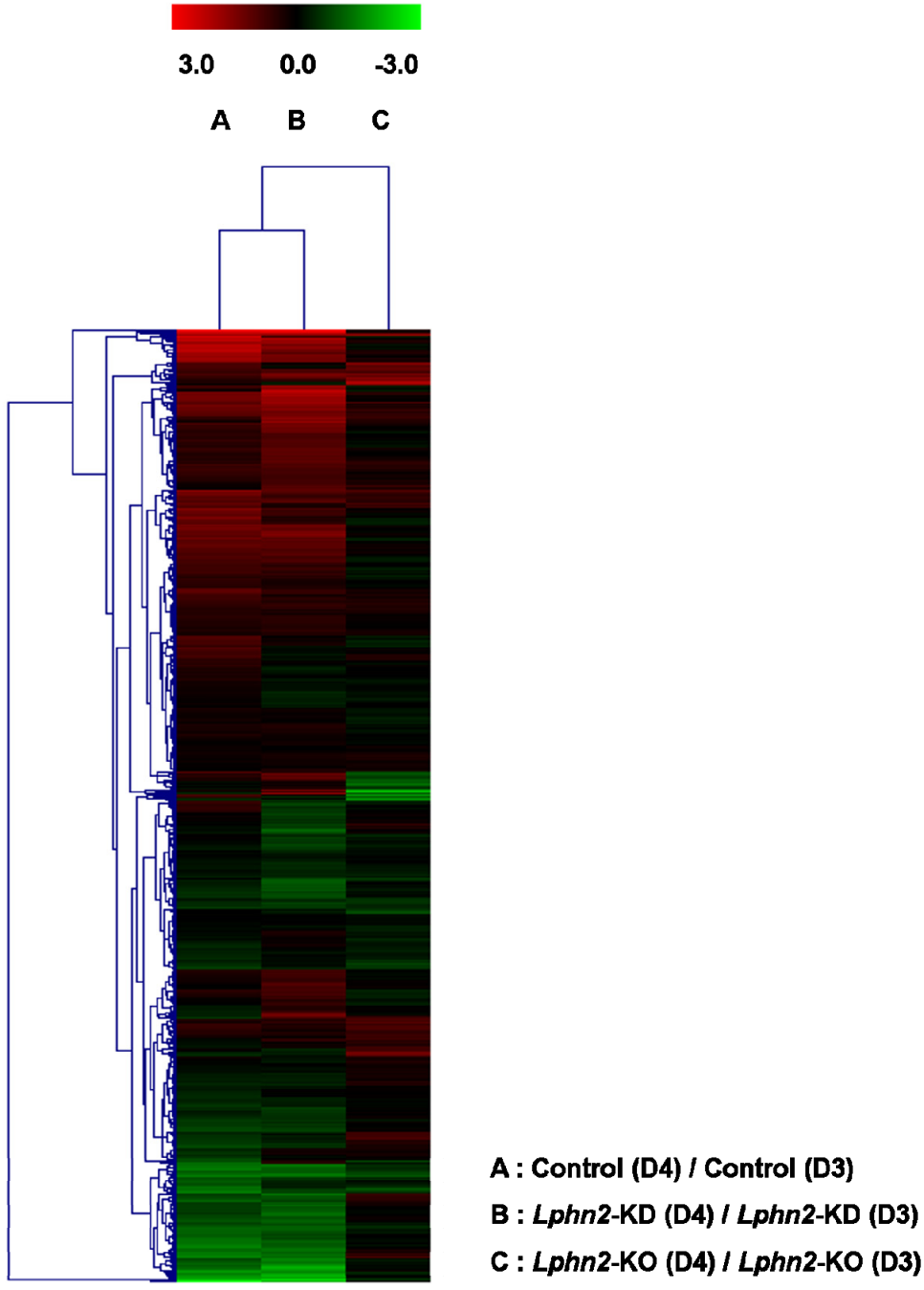

Figure S5 (Related to Figure 4). Heat map based on a Phospho Explorer Antibody Array. Differential phosphorylation ratio between groups A (control (D4)/control (D3)), B (*Lphn2*-KD (D4)/*Lphn2*-KD (D3)), and C (*Lphn2*-KO (D4)/*Lphn2*-KO (D3)).

Figure S6

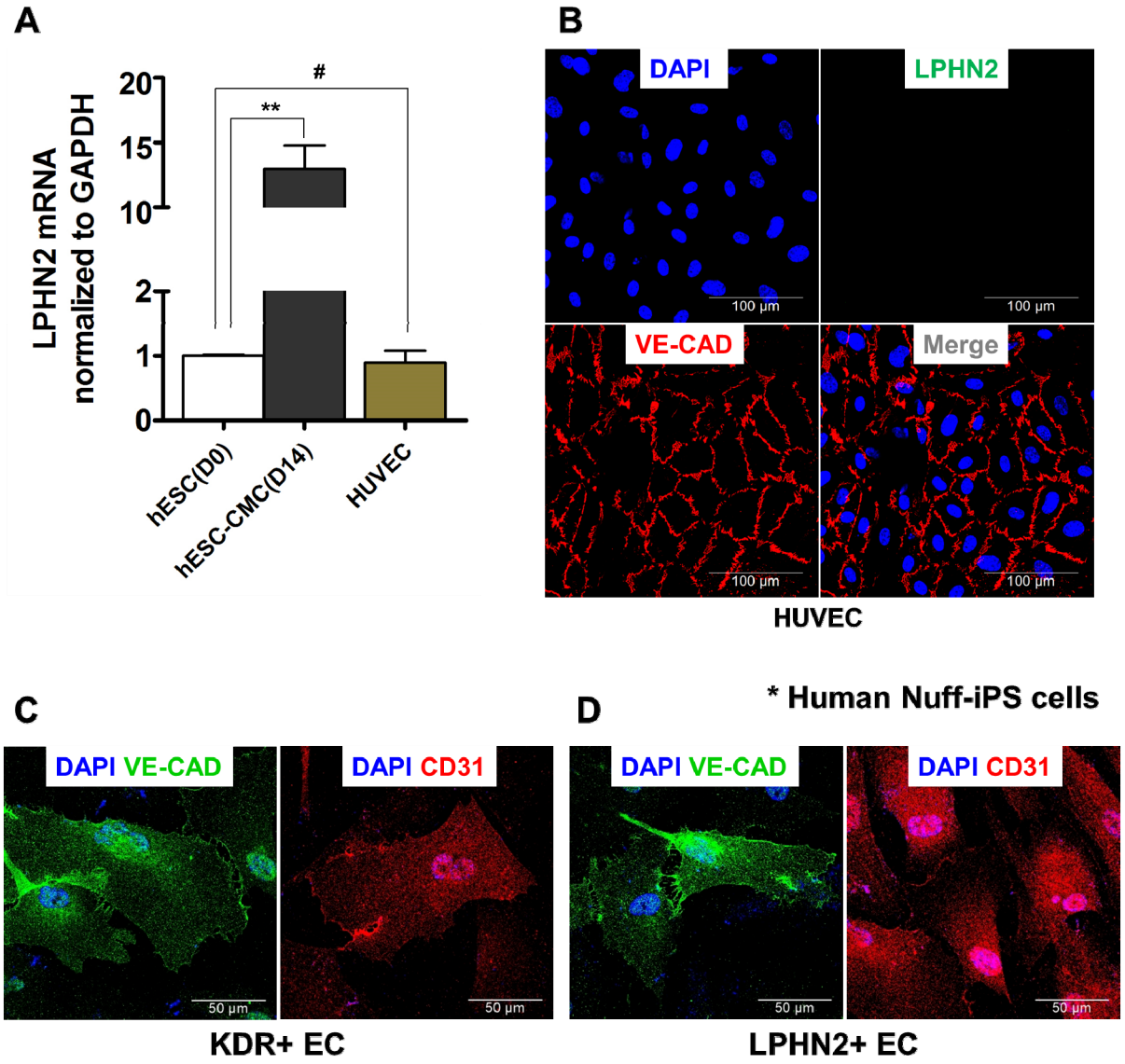

Figure S6 (Related to Figure 6). LPHN2 expression in HUVEC and endothelial differentiation potential of the KDR-positive or LPHN2-positive population.

(A) qPCR analysis of *LPHN2* expression in human ESCs, human ESC-derived CMCs and HUVECs. Expression values are shown relative to that of hESCs (D0). $^{**}P < 0.01$, #*P* = N.S. (not significant), ANOVA test and *post hoc* Bonferroni test, $n = 3$ independent replicates. **(B)** Immunostaining for LPHN2 (green) and VE-Cadherin (red) in HUVECs. Blue, nuclear counterstaining with 4, 6-diamidino-2-phenylindole (DAPI). Scale bar, 100 µm.

(C) Immunofluorescence analysis for EC markers (VE-Cadherin and CD31) in KDR^+ cells differentiated toward endothelial lineages on day 14. Blue, nuclear counterstaining with 4, 6-diamidino-2-phenylindole (DAPI). **(D)** Immunostaining of VE-Cadherin and CD31 in $LPHN2^+$ cells on day 14 of endothelial differentiation. Blue, nuclear counterstaining with 4, 6-diamidino-2-phenylindole (DAPI). Scale bar, 50 μm.

Supplemental Movie Legends

Movie S1 (Related to Figure 3). Beating foci following control-iPSCs and cardiac differentiation. On day 10 of cardiac differentiation, control-shRNA transduced iPSCs exhibited robust beating.

Movie S2 (Related to Figure 3). Beating foci following *Lphn2* knockdown (KD) and cardiac differentiation. On day 10 of cardiac differentiation, *Lphn2*-shRNA transduced iPSCs did not beat spontaneously.

Movie S3 (Related to Figure 3). Beating foci following wild-type ESCs and cardiac differentiation. On day 10 of cardiac differentiation, there were several spontaneous beating foci in wild-type ESCs.

Movie S4 (Related to Figure 3). Beating foci following *Lphn2* knockout (KO) and cardiac differentiation. On day 10 of cardiac differentiation, there were not spontaneous beating foci in *Lphn2*-KO ESCs.

Table S1. PCR primers used in this study

Primer	Sequence	Access Number
Mouse Lphn1	Forward 5′- AAGACATGAATGCCACCGAA -3′ Reverse 5′- CTCTTGGGGGAACACCAACT -3′	NM_181039.2
Mouse Lphn2	Forward 5′- GCTGCAAGCTGGTTGACACT -3′ Reverse 5′- GATGCAGATAGCCAGGCAGA -3’	NM_001081298.1
Mouse Lphn3	Forward 5′- TTTATAGGACCGGCGACCTT -3′ Reverse 5′- TACATGAGTCCAAAGGCCCA -3′	NM_198702.2
Mouse Nanog	Forward 5′- CAGAAAAACCAGTGGTTGAAGACTAG -3′ Reverse 5′- GCAATGGATGCTGGGATACTC -3	NM_001289828.1
Mouse Oct3/4	Forward 5′- GAGGATCACTTGGGGTACA -3′ Reverse 5′- CTCGAAGCGACAGATGGTG -3′	NM_013633.3
Mouse Flk-1	Forward 5′- GGCGGTGGTGACAGTATCTT -3′ Reverse 5′- CTCGGTGATGTACACGATGC -3′	NM_010612.2
Mouse PdgfR-α	Forward 5’- TCCATGCTAGACTCAGAAGTCA -3’ Reverse 5’- TCCCGGTGGACACAATTTTTC -3’	NM_001083316.2
Mouse Mesp1	Forward 5’- TGTACGCAGAAACAGCATCC -3’ Reverse 5’- TTGTCCCCTCCACTCTTCAG -3’	NM_008588.2
Mouse Nkx2.5	Forward 5’- GACAAAGCCGAGACGGATGG -3’ Reverse 5’- CTGTCGCTTGCACTTGTAGC -3’	NM_008700.2
Mouse Myh6	Forward 5’- ACGGTGACCATAAAGGAGGA -3’ Reverse 5’- TGTCCTCGATCTTGTCGAAC -3’	NM_001164171.1
Mouse cTnT (Tnnt2)	Forward 5′- CAGAGGAGGCCAACGTAGAAG -3′ Reverse 5′- CTCCATCGGGGATCTTGGGT -3’	NM_001130174.2
Mouse Tbx5	Forward 5′- GGAGCCTGATTCCAAAGACA -3′ Reverse 5′- TTCAGCCACAGTTCACGTTC -3′	NM_011537.3
Mouse Isl1	Forward 5′- CACTATTTGCCACCTAGCCAC -3′ Reverse 5′- AAATACTGATTACACTCCGCAC -3′	NM_021459.4
Mouse Cd31 (Pecam1)	Forward 5′- TGCAGGAGTCCTTCTCCACT -3′ Reverse 5′- ACGGTTTGATTCCACTTTGC -3’	NM_001032378.2
Mouse Gapdh	Forward 5′- GACCCCTTCATTGACCTCAAC -3′ Reverse 5′- CTTCTCCATGGTGGTGAAGA -3′	NM_001289726.1
Human LPHN2	Forward 5′- CTGGTTGCAGAATGCGAAGT -3′ Reverse 5′- CAAATCTTGTCATCCGTCCG -3	NM_001297704.1
Human GAPDH	Forward 5′- AACATCATCCCTGCCTCTAC -3′ Reverse 5′- CCCTGTTGCTGTAGCCAAAT -3′	NM_001256799.2

Table S2. Antibodies, Cytokines, and Reagents

Primary antibody	**Cat. #**	**Company**	**Application**
Anti-Mouse CD309 (FLK-1) Biotin	13-5821-82	eBioscience	FC
Anti-Mouse CD140a (PDGFR-α) APC	17-1401-81	eBioscience	FC
Anti-Human/Mouse OCT3/4 PE	12-5841-80	eBioscience	FC
Nanog	3580	cell signaling	FC
Oct3/4	sc-9081	Santa Cruz	IF
Nkx2.5	sc-8697	Santa Cruz	FC
Anti-Cardiac Troponin T	ab10214	Abcam	FC
Anti-Sarcomeric Alpha Actinin	ab9465	Abcam	FC, IF
Monoclonal Anti-α-Sarcomeric Actin	A2172	Sigma-Aldrich	IF
GFAP	sc-6170	Santa Cruz	FC
AFP	sc-15375	Santa Cruz	FC
PECAM-1 (CD31)	sc-1506-R	Santa Cruz	IF
VE-Cadherin	sc-6458	Santa Cruz	IF
Latrophilin-2	sc-47091	Santa Cruz	FC
Anti-LPHN2	ab101833	Abcam	IF
Phospho-CDK5 [Tyr15]	sc-12918	Santa Cruz	WB
Phospho-Src [Ser75]	Ab194520	Abcam	WB
Phospho-P38MAPK [Thr180/Tyr182]	9211	cell signaling	WB
CDK5	684502	BioLegend	WB
Src	2109	cell signaling	WB
P38MAPK	9212	cell signaling	WB
β-actin	sc-1615	Santa Cruz	WB
Anti-VEGF Receptor 2 (FLK-1)	ab10972	Abcam	IF
Lectin from Ulex europaeus (UEA-I)	L9006	Sigma-Aldrich	IF
Anti-human CD172a/b (SIRPα/β)	323804	BioLegend	FC
Human VCAM-1/CD106	FAB5649G	R&D systems	FC
Human ROR1	FAB2000G	R&D systems	FC
Human CD31	555446	BD Pharmingen	FC
Human CD144	17-1449-42	eBioscience	FC
Human PDGFR-α	FAB1264A	R&D systems	FC

Secondary Antibody	**Cat. #**	**Company**	**Application**
Streptavidin PE	12-4317-87	eBioscience	FC
Alexa Fluor 488 Donkey Anti-Goat IgG	A11055	Invitrogen	IF, FC
Alexa Fluor 488 Donkey anti-Rabbit IgG	A21206	Invitrogen	IF
Alexa Fluor 488 Goat Anti-Chicken IgG	A11039	Invitrogen	IF
Alexa Fluor 555 Goat Anti-Mouse IgG	A21422	Invitrogen	IF
Alexa Fluor 555 Donkey anti-Rabbit IgG	A31572	Invitrogen	IF
Alexa Fluor 555 Donkey Anti-Goat IgG	A21432	Invitrogen	IF
Alexa Fluor 555 Goat anti-Mouse IgM(μ chain)	A21426	Invitrogen	IF
Streptavidin, Alexa Fluor 555 Conjugate	S32355	Invitrogen	IF
Alexa Fluor 647 Donkey anti-Rabbit IgG	A31573	Invitrogen	IF, FC
Alexa Fluor 647 Donkey anti-Goat IgG	A21447	Invitrogen	IF, FC
Alexa Fluor 647 Donkey anti-Mouse IgG	A31571	Invitrogen	IF, FC
Alexa Fluor 647 Goat anti-Mouse IgM(μ chain)	A21238	Invitrogen	IF
Cytokines and Reagents	**Cat. #**	**Company**	
Activin A	338-AC	R&D Systems	
BMP-4	5020-BP	R&D Systems	
bFGF	13256029	Invitrogen	
EGF	236-EG	R&D Systems	
Cardiotrophin-1	612-CD	R&D Systems	
VEGF 164	493-MV	R&D Systems	
Recombinant human VEGF 165	293-VE-050	R&D Systems	
Recombinant human PDGF-BB	220-BB-010	R&D Systems	
Leukaemia Inhibitory Factor (LIF)	ESG1107	Millipore	
α-Latrotoxin	LSP-130	Alomone labs	
FLRT3	2795-FL	R&D Systems	

FC : Flow Cytometry, IF : ImmunoFluorescence, WB : Western Blot

Supplemental Experimental Procedures

RNA isolation and qPCR

Total RNA was prepared using QIAshredder and RNeasy Mini Kit (Qiagen, Inc.) according to the manufacturer's instructions. RNA (1 μg) was converted into cDNA using the ReverTra Ace® qPCR RT Master Mix (TOYOBO). qPCR was performed using FastStart Universal SYBR Green Master (Roche) with specific primers. Primer sequences are provided in Table S1. qPCR samples were run on an ABI PRISM-7500 sequence detection system (Applied Biosystems). Data are presented as relative quantification values. Gapdh was run simultaneously as a control and used for normalization.

Antibody array

1) Sample preparation: The protein was extracted by using protein extraction buffer (Fullmoon biosystems, Sunnyvale, CA) containing 1% protease inhibitor cocktail (Sigma, St. Louis, Mo) and 1% phosphotase inhibitor cocktail (Sigma, St. Louis, Mo) and lysis beads (Fullmoon biosystems, Sunnyvale, CA). After extraction, the protein solution was purified using gel matrix column that was included in antibody array assay kit (Fullmoon biosystems, Sunnyvale, CA). The column was vortex-mixed at 5 seconds and hydration-treated at 60 minutes on room temperature. After hydration, the column was centrifuged at 750 g for 2 minutes. After centrifuge, the column was placed into a collect tube and the 100 μl of protein sample was transferred into column. The column was centrifuged at 750 g for 2 minutes. The concentration of purified sample was measured with BCA protein assay kit (Pierce, Rockford, Ill) using NanoPhotometerTM (Implen, UK). Moreover, the purity of purified sample was confirmed on UV spectrum.

2) Phospho Explorer Antibody Array: The 50 μg of protein sample was filled up 75 μl with labeling buffer and treated 3 μl of the 10 μg/μl biotin/DMF solution. The sample was incubated at room temperature for 90 min with mixing. After incubation, the sample was treated 35 μl of stop reagent and incubated at room temperature for 30 min with mixing. The antibody microarray slide (Fullmoon biosystems, Sunnyvale, CA) was treated 30 ml of blocking solution in a petri dish and incubated on shaker at 60 rpm for 30 min at room temperature and washed with distilled water. This step was replicated three times. After blocking, the slide was rinsed with Milli-Q grade water. The labeled sample was mixed in 6 ml of coupling solution. The blocked array slide was incubated with coupling mixture on shaker at 60 rpm for 2 hours at room temperature into coupling dish. After coupling, the slide was washed 6 times with 30 ml of washing solution into petri dish on shaker at 60 rpm for 5 minutes. Next, the slide rinsed with Milli-Q grade. The 30 μl of 0.5 mg/ml Cy3-streptavidin (GE Healthcare, Chalfont St. Giles, UK) was mixed in 30 ml of detection buffer. The coupled array slide was treated with detection mixture into petri dish on shaker at 60 rpm for 20 minutes at room temperature. After detecting, the slide was washed 6 times with 30 ml of washing solution into petri dish on shaker at 60 rpm for 5 minutes. Next, the slide rinsed with Milli-Q grade water.

3) Data acquisition and analysis: The slide scanning was performed using GenePix 4100A scanner (Axon Instrument, USA). The slides were absolutely dried before the scanning and scanned within 24~48 hours. The slides were scanned at 10 μm resolution, optimal laser power and PMT. After got the scan image, they were grided and quantified with GenePix 7.0 Software (Axon Instrument, USA). The normalization data were analyzed using Genowiz 4.0TM (Ocimum Biosolutions, India). This normalization makes the average intensity of all samples numerically equivalent to the average intensity of the all genes. Moreover, the *p*-value (one-

sample *t*-test) was calculated using MeV 4.9.0 Software (TM4 Development Group, USA). After analyzing, the data about protein information was annotated using UniProt DB. The phosphorylation ratio was calculated using the following formula (phosphorylated and matching unphosphorylated values are denoted by phospho and unphospho in both the differentiation day 3 and day 4).

Phosphorylation ratio = (phospho of differentiation day 4/unphospho of day 4) / (phospho of differentiation day 3/ unphospho of day 3)

Antibody array results are accessible at the GEO database (accession number, GSE92923).

Western blot

Cells were harvested and lysed for 30 minutes in lysis buffer containing protease inhibitors (Roche, Basel, Switzerland). Total protein (40 μg) was immunoblotted with primary antibodies against phospho-CDK5 [Tyr15] (Santa Cruz Biotechnology, Dallas, TX, USA; goat polyclonal; molecular weight = 35 kDa), phospho-Src [Ser75] (Abcam, Cambridge, UK; rabbit polyclonal; molecular weight = 60 kDa), phospho-P38MAPK [Thr180/Tyr182] (Cell Signaling, Danvers, MA, USA; rabbit polyclonal; molecular weight = 43 kDa), total-CDK5 (BioLegend, San Diego, USA; mouse monoclonal; molecular weight = 35 kDa), total-Src (Cell Signaling, Danvers, MA, USA; rabbit monoclonal; molecular weight = 60 kDa), total-P38MAPK (Cell Signaling, Danvers, MA, USA; rabbit polyclonal; molecular weight = 43 kDa), and β-actin (Santa Cruz Biotechnology, Dallas, TX, USA; goat polyclonal; molecular weight = 43 kDa). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (for phospho-Src, and phospho-P38MAPK, total-Src, total-P38MAPK; Santa Cruz Biotechnology, Dallas, TX, USA), anti-goat IgG (for phospho-CDK5 and β-actin; Santa Cruz Biotechnology, Dallas, TX, USA) and anti-mouse IgG (for total-

CDK5; Santa Cruz Biotechnology, Dallas, TX, USA) antibodies were used as secondary antibodies. Amersham ECL western blotting detection reagents (GE Healthcare Life Sciences, Chicago, IL, USA) were used for detection. Quantification of band intensity was analyzed using TINA software, version 2.0 (RayTest, Straubenhardt, Germany), and was normalized to the total-form antibody.

Endothelial and smooth muscle differentiation of Human PSC

For endothelial and smooth muscle differentiation of human PSCs, we optimized using the previously described protocols. Human PSCs were directed towards the cardiac progenitor cells until day 5. After day 5 sorting, KDR^+ or $LPHN2^+$ cells were induced in EGM-2MV medium (Cat. #: CC-3124, LONZA) supplemented with 50 ng/ml VEGF and 5 μM SB431542 for endothelial, or 8 ng/ml PDGF-BB and 10 ng/ml bFGF for smooth muscle differentiation.

Immunofluorescence staining

Cells were fixed with 2% paraformaldehyde (PFA, Wako) for 10 minutes at room temperature. After being washed with PBS and blocked with PBS containing 0.05% Triton-X100 and 1% BSA, the cells were incubated with primary antibodies for 18 hrs at 4°C. After washing, corresponding secondary antibodies were applied for 1 h at room temperature. The nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) and mounted using the fluorescent mounting medium (DAKO). Fluorescent images were acquired with an LSM710 confocal microscope (Zeiss) and a confocal microscope Leica TCS SP8 (Leica Microsystems, Wetzlar, Germany). Information of the primary and secondary antibodies for immunofluorescence is provided in Table S2.

Flow cytometric analysis and cell sorting

EBs were harvested and treated with Accutase (eBioscience) for 5 min at 37°C to dissociate cells. Cells were then washed with PBS, stained with specific antibodies (Table S2) for lineage markers. The fixation and permeabilization buffer kit (R&D system) was used as recommended in the manufacturer's instructions for intracellular staining of OCT3/4, NANOG, cTNT, α-SA, NKX2.5, GFAP, and AFP. Analysis of stained cells and sorting were performed by flow cytometry using fluorescence-activated cell sorting (FACS) Canto II TM or Aria II TM (BD Biosciences) and obtained data were analyzed by FlowJo (Tree Star, Ashland, OR, USA).

Lentiviral transduction

For *Lphn2* knockdown in iPSCs, five shRNA constructs targeting different regions of the *Lphn2* gene were used (Sigma-Aldrich, SHCLNV, MISSION® shRNA Lentiviral Transduction Particles, TRCN0000238691, TRCN0000238692, TRCN0000238693, TRCN0000238694, TRCN0000238695). Non-Target shRNA Control Transduction Particles (Sigma-Aldrich, SHC216V) was used as the shRNA negative control. Transduction was performed according to manufacturer's instructions. At 48 hrs after transduction, protein-iPSCs were selected in growth medium containing puromycin (5 μg/ml, Sigma-Aldrich). Transduced iPSCs were analyzed for *Lphn2* knockdown efficiency using qPCR.

Lphn2-KO ESCs

Lphn2-KO ESCs ($Lphn2^{tm1a\ (EUCOMM)\ Hmgu}$, EUCOMM) were purchased from EUCOMM. *Lphn2*-KO ESCs were cultured in knockout DMEM (Gibco) including 10% fetal bovine serum,

0.1 mM β-mercaptoethanol (Sigma-Aldrich, filter sterilized), 2 mM L-glutamine, 50 IU/mL penicillin, 50 mg/mL streptomycin (Gibco), and LIF (2000 U/mL) on MEF as feeder-layer cells.