Stem Cell Reports

Supplemental Information

Repair of Ischemic Injury by Pluripotent Stem Cell Based Cell Therapy without Teratoma through Selective Photosensitivity

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

ES cell culture

The mouse J1 cell line was purchased from the American Tissue Culture Collection (ATCC, VA, USA). To maintain the cells in an embryonic state, they were cultured on a feeder free in mESC medium (DMEM (Gibco, Life technologies, NY, USA) supplemented with 15% fetal bovine serum (FBS) (Gibco), 1% nonessential amino acids (Gibco), 0.1 mM β-mercaptoethanol (Gibco), 0.1% gentamycin (Gibco) and 1,000 U/ml mouse leukemia inhibitory factor (mLIF) (Millipore, Merck, Germany) in a humidified atmosphere at 37°C with 5% CO_2. The medium was changed daily, and the cells were passaged every 2~3 days. Human ESCs (H9: Wicell Research Institute, Madison) were maintained in TeSR^{TM}-E8^{TM} medium (Stemcell Technologies, Canada) on matrigel (BD Biosciences, NJ, USA)-coated 60-mm dishes.

Spontaneous differentiation

To induce differentiation of mESCs, they were allowed to form mEB in suspension culture conditions in mESC medium without mLIF for 3 days. EB media was changed every 2 days. mEBs were then plated on 1% gelatin-coated plates and cultured in mESC medium without mLIF for 12 days. For rapid hESC differentiation, hESCs were cultured in media containing 10% FBS for 7 days on matrigel-coated dishes.

Annexin V staining

mESCs (Mock and KR) and miPSCs (Mock and KR) were plated at 2×10^5 cells/well of gelatinized 24-well plate and cultured for 24 hours in standard ES cell medium. hESCs (GFP and KR) were cultured in standard medium for appropriate time. Cells were exposed by light of 540~580 nm and incubated for 24 hours. Cells were stained

using the PE Annexin V apoptosis detection kit I (BD Pharmingen, CA, USA) according to the manufacturer's instructions. Annexin V^+ cells were gated as dead cells. Gating Annexin V^- population was used as a negative control. The cell death percentage was determined by flow cytometric analysis.

Morphological analyze for cell death

mESCs (Mock and KR) were exposed by light of 540 nm for an appropriate time. Cell death kinetics of mESCs (Mock and KR) cells was recorded using a JuLI Smart fluorescent cell analyzer microscope (Montreal Biotechnologies Inc. (MBI), Dorval, PQ, Canada) for approximately 24 hours. For mix populations, EC-KR-mESCs (2×10^5 cells/well) were previously plated on gelatinized 24-well plate and incubated at 37°C for 24 hours. KR-mESCs were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Life Technologies), fluorescein-derived intracellular green fluorescent label, to distinguish undifferentiated KR-mESCs from mixed cells. CFSE staining performed according to the manufacturer's instructions. CFSE-stained KR-mESCs were plated on EC-KR-mESCs plated 24-well plate. Mixed cell populations were exposed to light of 540 nm and analyzed morphological change for their apoptotic response by JuLI Smart fluorescent cell analyzer microscope (Montreal Biotechnologies Inc.).

Trypan blue assay

Mock- and KR-mESCs were exposed to light of 540 nm for proper time and incubated for 24 hours. The cells were stained with 0.4% trypan blue dye and then counted using a hematocytometer. Each experiment was conducted in triplicate and the results were expressed as the mean ± SEM for each group.

Immunoblotting

Cells were lysed with TLB buffer (20 mM Tris-HCl (pH7.4), 137 mM NaCl, 2 mM EDTA, 1% triton X-100, and 10% glycerol) supplemented with 10 uM sodium vanadate and 1 mM protease inhibitor cocktail (Roche, Basel, Switzerland) and subjected to SDS-PAGE followed by immunoblotting using primary antibodies KillerRed (Evrogen, Russia), cleaved caspase-3 (9664S), OCT-4 (2840P), SOX2 (14962P), and NANOG (4903P) (Cell signaling, Danvers, MA), PARP (sc-7150) , and ERK2 (sc-154) (Santa Cruz Biotech Inc., Dallas, TX), and horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA).

Caspase-3 activity assay

The Ac-DEVD-AMC caspase-3 fluorogenic substrate (BD Pharmingen) was used for assays performed according to the manufacturer's instructions.

Total RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted from the cells using easy-BLUE^{TM} Total RNA Extraction Kit (Intron, Seongnam, South Korea) in accordance with the manufacturer's instruction. The extracted total RNA was converted to cDNA using PrimeScript^{TM} RT Master Mix (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instruction. Gene-specific primers were as follows: *mOct-4* (forward: 5'-GAGAAAGCGAACTAGCATTGAGAAC-3', reverse: 5'-TGTAGCCTCATACTCTTCTCGTTG-3'); *hOCT-4* (forward: 5'-CCCCAGGGCCCCATTTTGGTACG-3', reverse: 5'-ACCTCAGTTTGAATGCATGGGAGAGC-3'); *mNanog* (forward: 5'-

GTGCACTCAAGGACAGGTTTCAG-3', reverse: 5'-CTGCAATGGATGCTGGGATACTC-3'); *hNANOG* (forward: 5'-AAATTGGTGATGAAGATGTATTCG-3', reverse: 5'-GCAAAACAGAGCCAAAAACG-3'); *mSox2* (forward: 5'-ATGGGCTCTGTGGTCAAGTC-3', reverse: 5'-CCCTCCCAATTCCCTTGTAT-3'); *hSOX2* (forward: 5'-TTCACATGTCCCAGCACTACCAGA-3', reverse: 5'-TCACATGTGTGAGAGGGGCAGTGTGC-3'); *KillerRed* (forward: 5'-CAACGAGACCCACATGTTCC-3', reverse: 5'-CTGGTGTCCCTCATCTGCTT-3'); *mBrachyury(T)* (forward: 5'-CATCTGCTTGTCTGTCCATGCTG-3', reverse: 5'-GAGAACCAGAAGACGAGGACGTG-3'); *mFgf5* (forward: 5'-CATCGGTTTCCATCTGCAGATCTAC-3', reverse: 5'-GTTCTGTGGATCGCGGACGCATAG-3'); *mSox17* (forward: 5'-ACCCAGATCTGCACAACGCAGAG-3', reverse: 5'-GCTTCATGCGCTTCACCTGCTTG-3'); and *mGapdh* (forward: 5'-TCTGGAAAGCTGTGGCGTGATGG-3', reverse: 5'-CAGATGCCTGCTTCACCACCTTC-3'). The amplified template was detected using SYBR® Premix Ex Taq™ (Takara Bio Inc.) with a real-time PCR system (LightCycler® 480; Roche, Basel, Switzerland). PCR was conducted in 20 ul of reaction volume containing 10 ul SYBR green mix (Applied Biosystems, Foster, CA), 0.1 uM of each primer, and 2 ul of cDNA template with the following conditions: 95°C for 30 sec for denaturation of template, and 40 cycles of 95°C for 5 sec, 58°C for 15 sec, and 72°C for 20 sec.

ROS detection

Intracellular hydroperoxide and superoxide anion production were determined by

flow cytometry using DCF-DA as fluorescent probes. DCF-DA fluorescent signal from KR-mEDSCs with no light exposure was used as a negative control. KR-mESCs were exposed by light of 540~580 nm and incubated for 2 hours. The cells were then also incubated with the probes (10 uM) in HBSS (Gibco) for 30 min at 37°C, after which they were washed, and analyzed for fluorescence intensity using fluorescence microscope (Olympus Corporation, Tokyo, Japan) and FACS Caliber (BD Biosciences, Bedford, MA). Higher DCF-DA signal than negative control was gated as a positive population that produced ROS.

Immunocytochemistry

KR-mESCs and endothelial cells derived from KR-mESCs (ECs-KR-mESCs) were fixed in 4% PFA (paraformaldehyde) for 20 minutes and permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. After treatment, 5% normal goat serum (blocking solution) was applied for 30 minutes and the cells were incubated with primary antibodies OCT-4 (2840P), cleaved caspase-3 (9664S) (Cell signaling), SSEA-1 (nb 100-1831, Novus Biologicals, Littleton, CO), and vWF (ab11713, Abcam, Cambridge, MA) in blocking solution overnight at 4°C. The cells were then washed three times with PBS and incubated with Cy^{TM}2 (711-225-152, Jackson ImmunoResearch Laboratories), Alexa Fluor® 488 (A-21200), and Alexa Fluor® 594 (A-21201) (Molecular probe Inc., Eugene, OR) conjugated secondary antibodies for 1 hour The images were analyzed using fluorescence microscopy (Olympus and Nikon, Eclipse Ti, Japan).

Differentiation and cell sorting

To induce endothelial cells, KR-mESCs were allowed to form mEB in suspension culture conditions in DMEM (Gibco) containing 5% FBS (Hyclone), 20 ng/ml mouse

recombinant vascular endothelial growth factor; VEGF (493-MV, R&D system Inc., Minneapolis, MN) with basic medium components for 10 days. EB media was changed every 2 days for 10 days. mEBs were then plated on 0.2% gelatin coated plates and cultured in DMEM supplemented with 5% FBS for 7 days. To isolate ECs from differentiated mEBs, cell sorting was performed with a FACS Aria 3 cell sorter (BD Biosciences) using APC-conjugated anti-mouse CD31 antibody (12-0311-82, eBioscience, CA, USA). APC-conjugated anti-mouse IgG (340754, BD Biosciences) was used as a negative control. CD31 positive cells were cultured on the 0.2% gelatin coated plates with DMEM containing 5% FBS, 20 ng/ml VEGF with basic medium components, and expanded for 2 passages.

Doppler analysis

Laser doppler imaging analysis was performed as previously described (18) A laser doppler perfusion imager (Moor Instruments, Devon, UK) was used to measure blood flow in the hind limbs on days 0, 3, 7, 14 and 28 post cell treatment. The digital color-coded images were analyzed to quantify the blood flow in the region from the knee joint to the toe, and the mean perfusion values were calculated.

Histology for ischemic tissues

For tissue staining, the specimens were fixed in 4% paraformaldehyde for 4 week. The sample was dehydrated with a series of graded ethanol, embedded in paraffin and serial sectioned at a thickness of 5 μm then stained with hematoxylin and eosin (H&E). Masson's trichrome collagen staining was performed to assess the existent of fibrosis in the ischemic tissue. To detect the capillary expression, tissue 5-μm serial sectioned tissues were stained with PECAM (CBL1337, Millipore) primary antibodies. The staining signal for PECAM was visualized with avidinebiotin

complex immunoperoxidase (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) and 3,30-diaminobenzidine substrate solution kits (Vector Laboratories)

Figure S1 (A) Sections of teratomas generated by KR-mESCs are shown stained with H&E, Masson's trichrome, and Alcian Blue. Teratoma produced cells indicative of gut for endoderm, adipose tissue, cartilage, muscle fiber, and connective tissue for mesoderm, neural rosette, keratin pearl, and squamous tissue for ectoderm. (Scale bar, 50 μm) (B) Phase contrast images (Phase contrast) and fluorescence microscopic images (RFP) of control miPSCs (Mock-miPSCs) and KR-miPSCs (Scar bar, 200 μm) (C) Sections of teratomas generated by KR-miPSCs are shown. (Scale bar, 50 μm)

Figure S2 (A) Immunoblotting analysis for KR and OCT-4 in KR-mESCs differentiated with monolayer-culture for 14 days after 3 days of EB formation for spontaneous differentiation. PCNA is for equal protein loading control. (B) Quantitative data of apoptotic cell death by Annexin V staining represent mean ± SD of experiments performed in two independent studies. (C) Immunoblotting analysis for cleaved caspase-3. ERK2 is for equal protein loading control.

Figure S3 (A) $CD31^+$ cell sorting from endothelial differentiation of KR-mESCs and characterization of sorted $CD31^+$ cells as EC-KR-mESCs by morphology and immunostaining of vWF (Scale bar, 100 μm (left) and 20 μm (right)) (B) Characterization of $CD31^+$ EC-KR-mESCs with tubule formation assay (top panel) and acetylated low-density lipoprotein (ac-LDL, Red) uptake assay (bottom panel) (Scale bars, 1000 μm (top) and 100 μm (bottom) respectively) (C) Fluorescence images of normal limbs and ischemic limbs with or without DiI (red) labeled EC-KR-mESCs transplantation, 24 days after treatment, DAPI staining (blue) for identifying the nucleus of cells (Scale bar, 100 μm) (D) Fluorescence images of ischemic limbs after transplantation of DiI (red) labeled EC-KR-mESCs with (bottom panels) or without light exposure (top panels), SMA (green) and DAPI (blue) staining

identifying recruited ECs and nucleus respectively, Open arrows for SMA positive area and closed arrows for Dil/SMA dual positive area, Scale bar, 100 μm

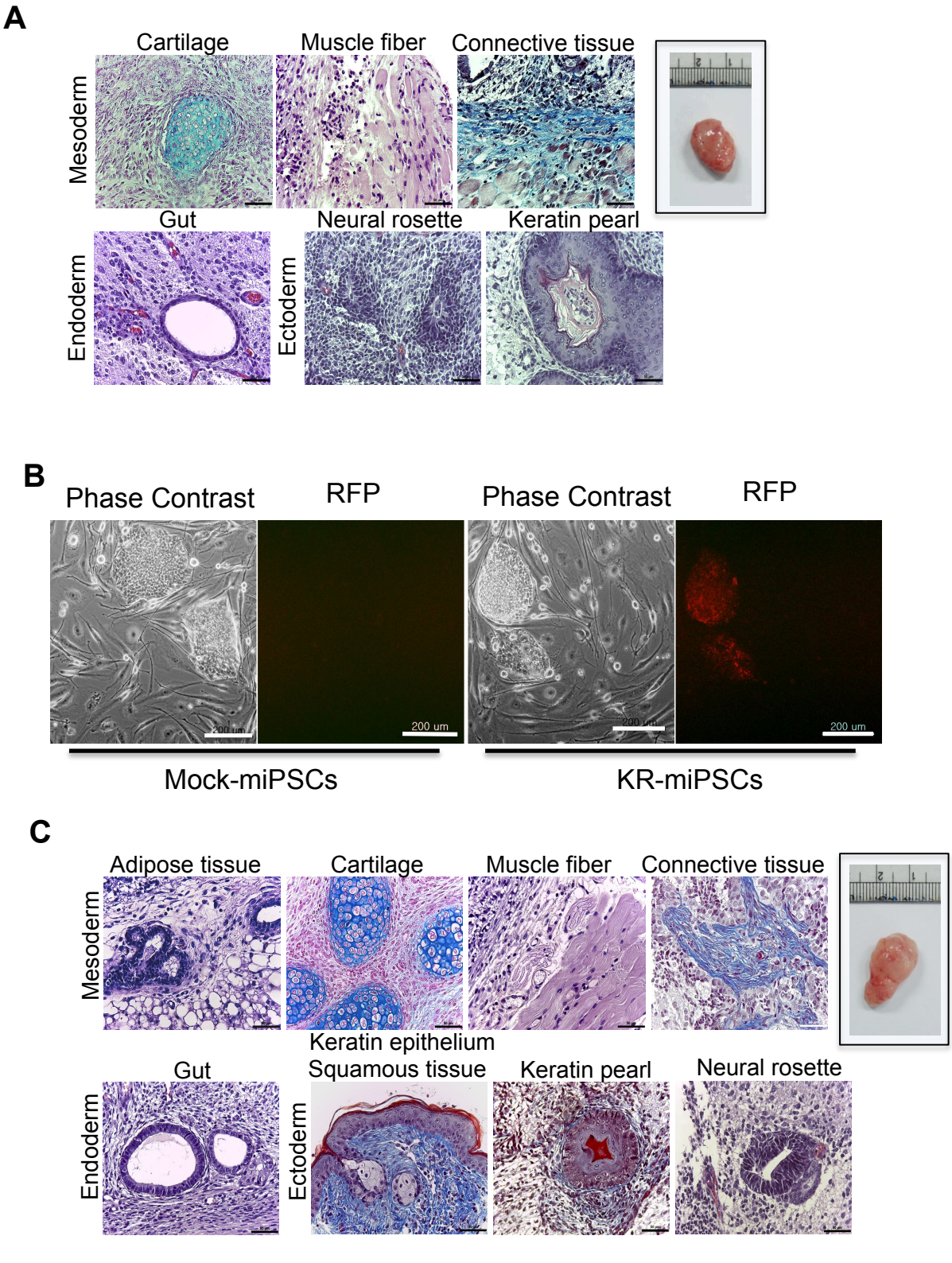
Figure S4 Analysis of all three germ-layer tissues within a formed teratoma after transplantation of mixed population (KR-mESCs and EC-KR-mESCs). (Scale bar, 100 μm)

Figure S5 All images of mice and teratomas developed from KR-miPSCs with (0/12: zero out of twelve mice) and without (14/14: fourteen out of fourteen mice) light exposure

Figure S6. All images of mice and teratomas from developed from transplantation of mixed population (KR-mESCs and EC-KR-mESCs) with (0/10: zero out of ten mice) and without (10/10: ten out of ten mice) light exposure

Figure S7. Characteristics of KR-hESCs and degradation of KR in human ESCs (A) Comparison of pluripotency markers (*hOCT-4*, *hSOX2 and hNANOG*) expression between GFP and KR-hESCs by quantitative real-time PCR analysis (B) Spontaneous differentiation of KR-hESCs for indicative days with FBS-contained medium in the absence of bFGF2. Results in A and B represent one of the experiments performed twice in duplicate plates. (C and D) Comparison of KR mRNA expression between established KR-hESCs and KR-mESCs by quantitative PCR (C) and immunoblotting analysis (D) Quantitative data represent mean ± SD of experiments performed in two independent studies (SE, short exposure; LE, long exposure) (E) Established hESCs (GFP and KR) were treated with 125 nM of MG-132 for 1 hour, and KR protein levels were determined by immunoblotting analysis. α-tubulin was used as a loading control.

Figure. S1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Cartilage
Muscle fiber
Connective tissue
Mesoderm
Gut
Neural rosette
Keratin pearl
Endoderm
Ectoderm
B
Phase Contrast
RFP
Phase Contrast
RFP
200 um
200 um
200 um
200 um
Mock-miPSCs
KR-miPSCs
C
Adipose tissue
Cartilage
Muscle fiber
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Gut
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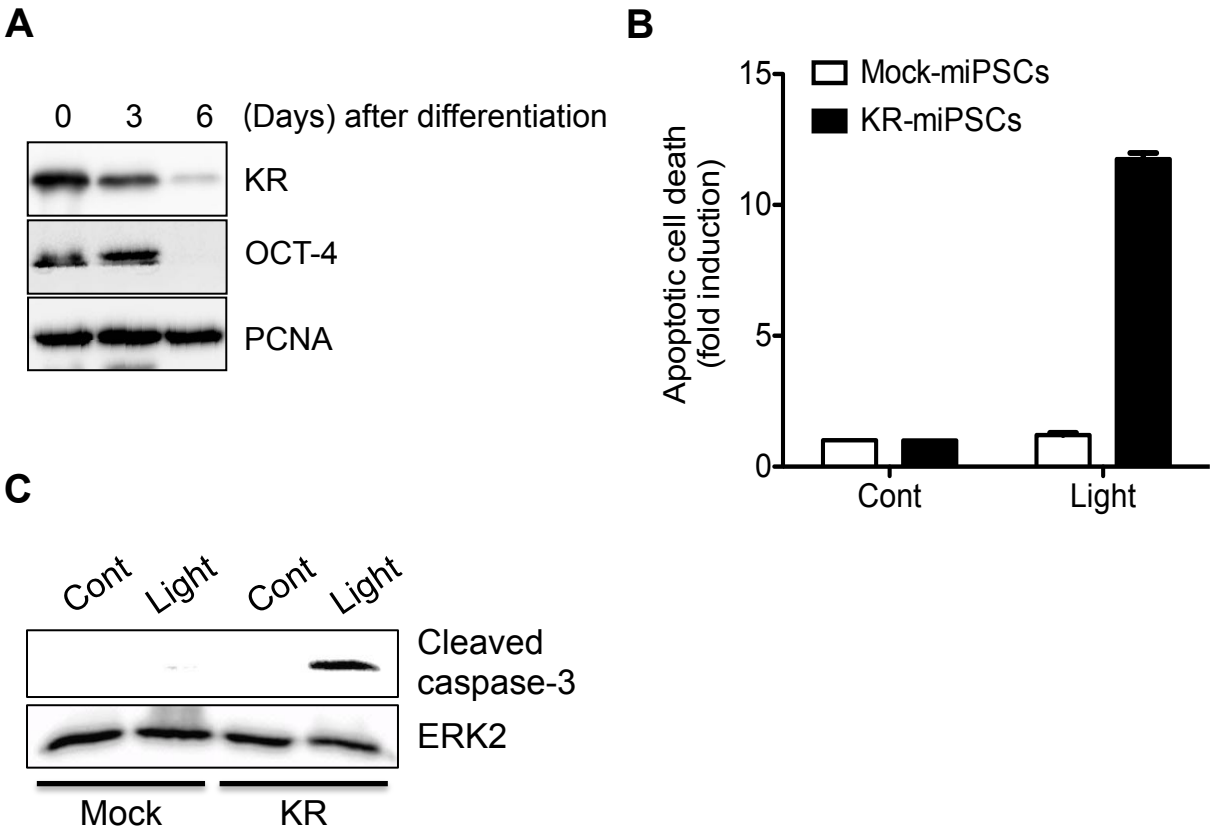
Endoderm
Ectoderm

Figure. S2


A
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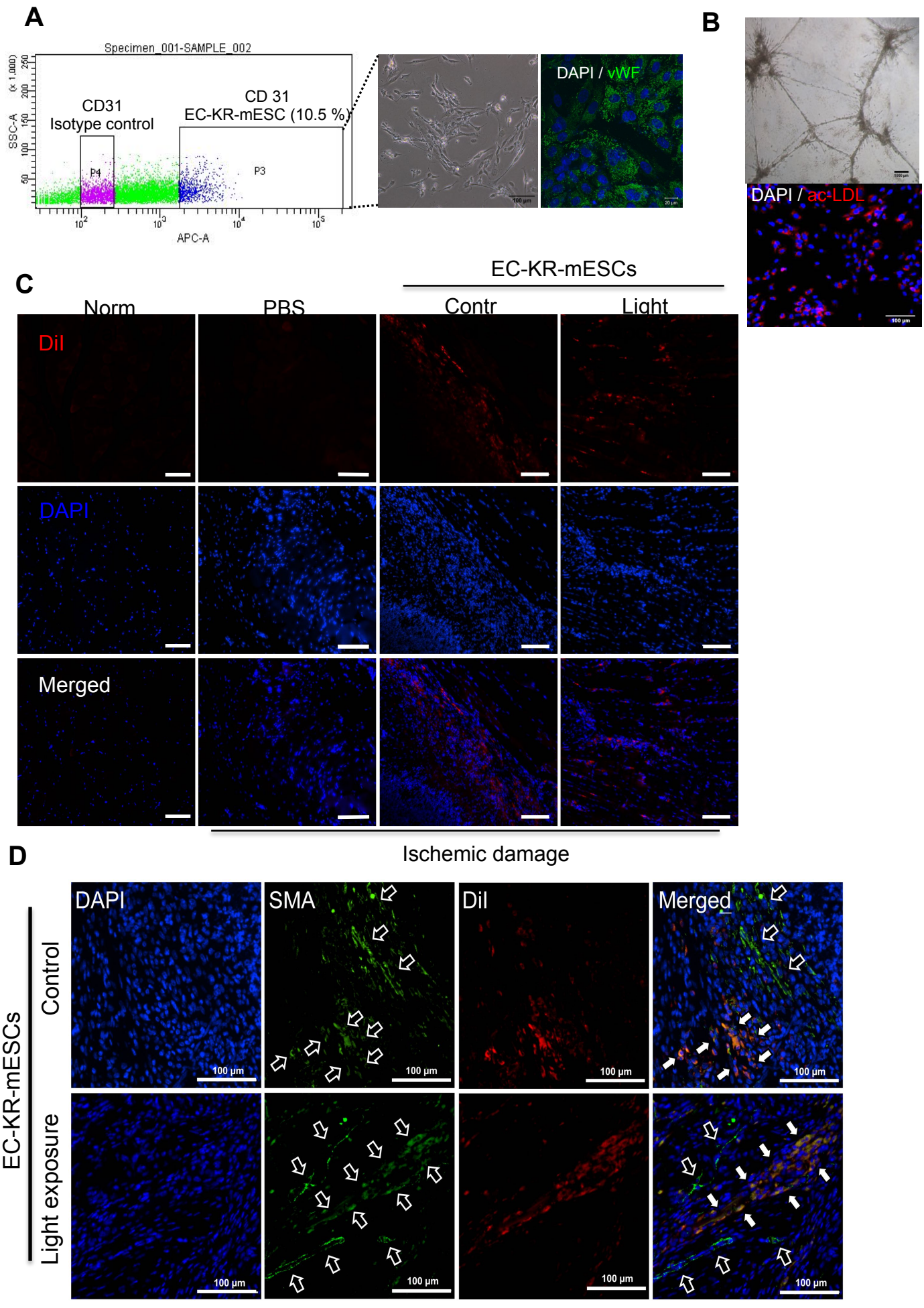
3
6
(Days) after differentiation
KR
OCT-4
PCNA
B
Mock-miPSCs
KR-miPSCs
Apoptotic cell death
(fold induction)
15
10
5
0
Cont
Light
C
Cont
Light
Cont
Light
Cleaved
caspase-3
ERK2
Mock
KR

Figure. S3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CD 31
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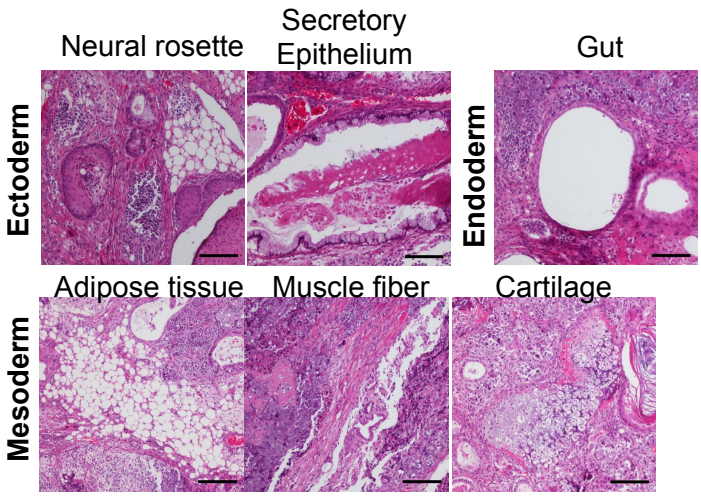
P3
SSC-A
APC-A
DAPI / vWF
B
DAPI / ac-LDL
C
EC-KR-mESCs
Norm
PBS
Contr
Light
DiI
DAPI
Merged
D
Ischemic damage
EC-KR-mESCs
Control
Light exposure
DAPI
SMA
DiI
Merged
100 μm

Figure. S4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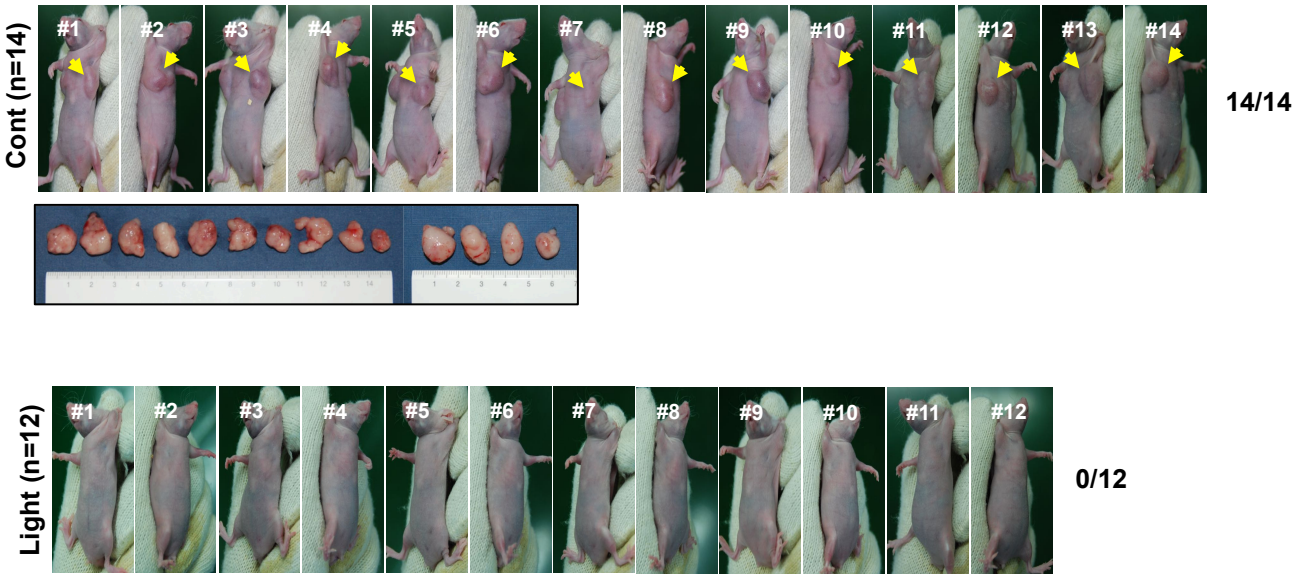
Secretory Epithelium
Gut
Ectoderm
Endoderm
Adipose tissue
Muscle fiber
Cartilage
Mesoderm

Figure. S5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#2
#3
#4
#5
#6
#7
#8
#9
#10
#11
#12
#13
#14
14/14
Light (n=12)
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
#11
#12
0/12

Figure. S6

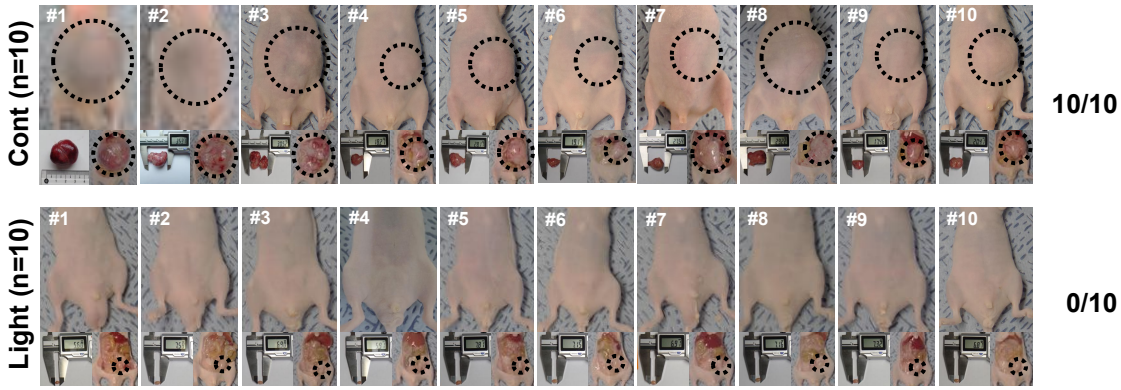
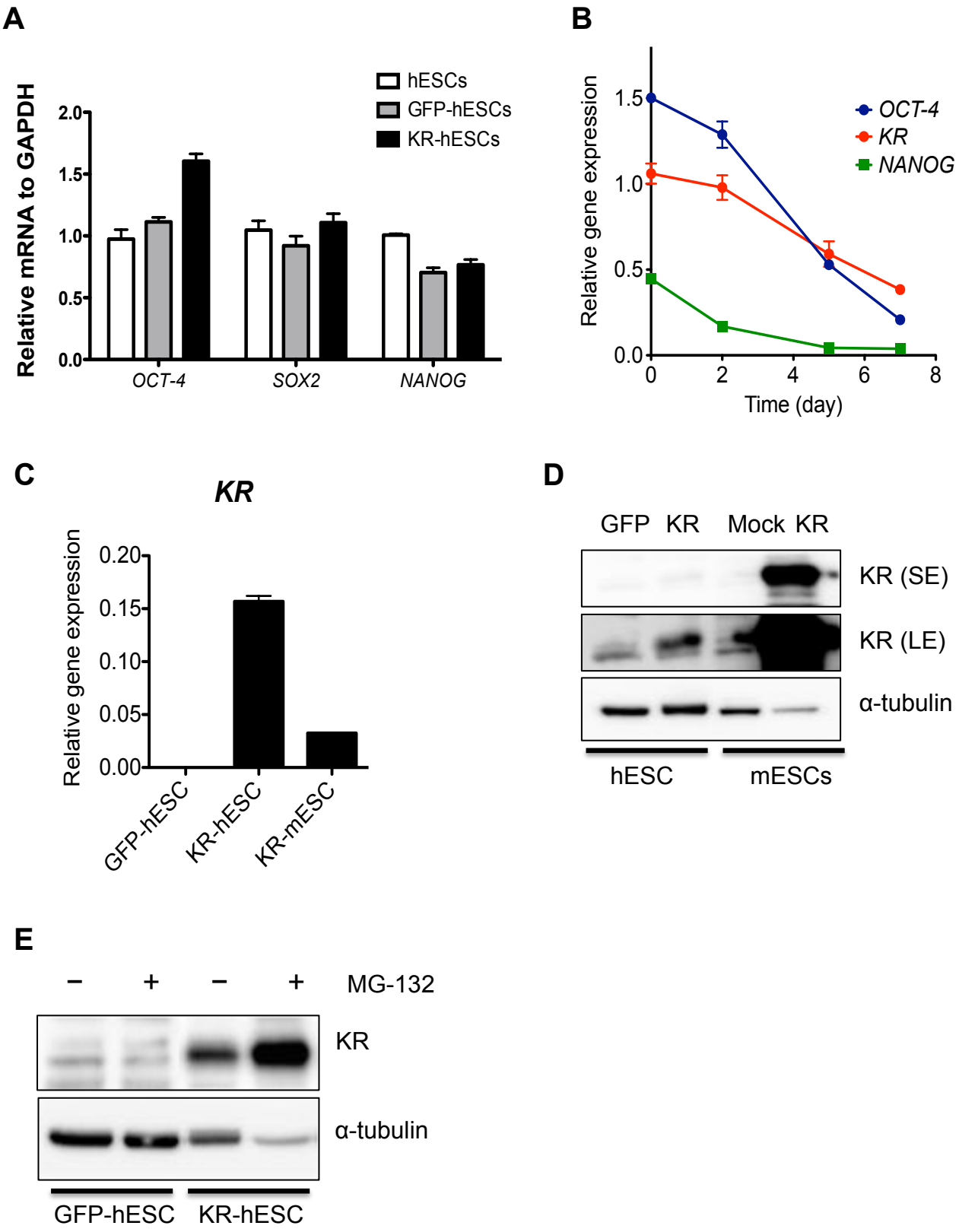


Figure. S7


A
Relative mRNA to GAPDH
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1.5
1.0
0.5
0.0
hESCs
GFP-hESCs
KR-hESCs
OCT-4
SOX2
NANOG
B
Relative gene expression
1.5
1.0
0.5
0.0
0
2
4
6
8
Time (day)
OCT-4
KR
NANOG
C
KR
Relative gene expression
0.20
0.15
0.10
0.05
0.00
GFP-hESC
KR-hESC
KR-mESC
D
GFP
KR
Mock
KR
KR (SE)
KR (LE)
α-tubulin
hESC
mESCs
E
−
+
−
+
MG-132
KR
α-tubulin
GFP-hESC
KR-hESC