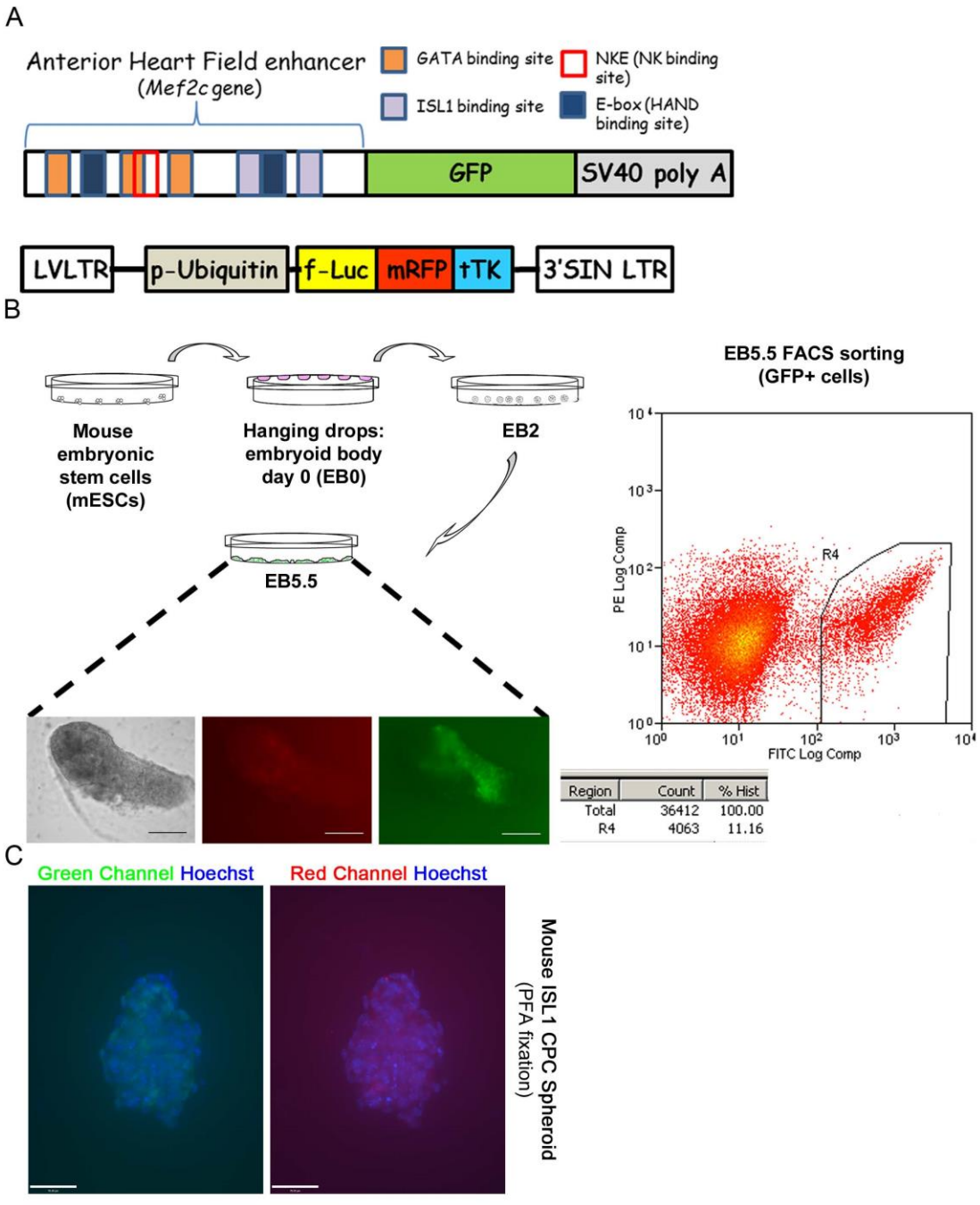
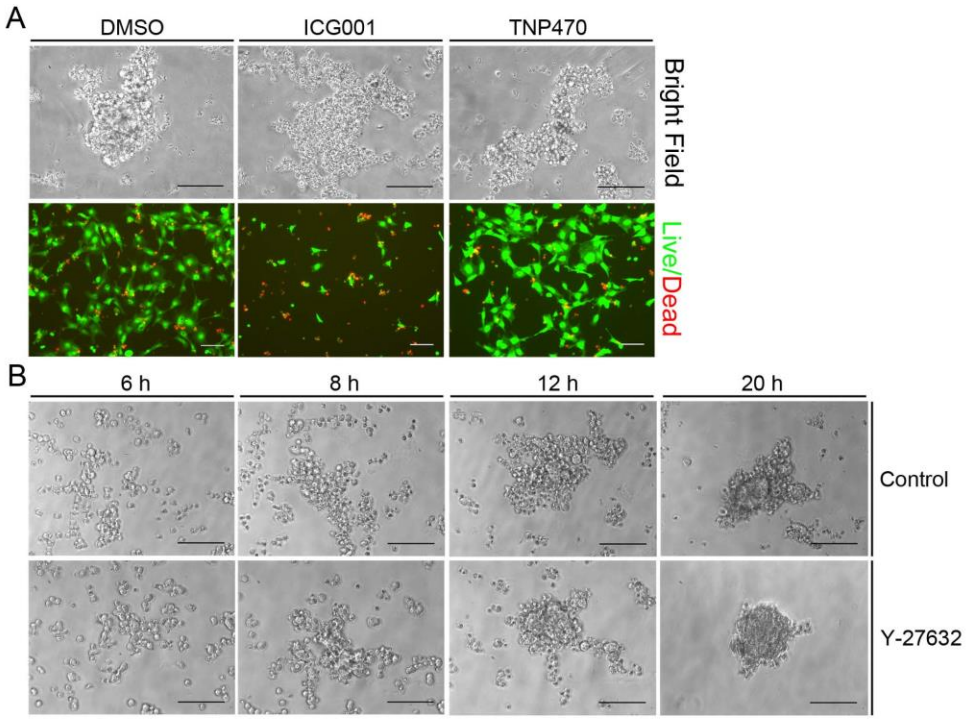
SUPPLEMENTAL DATA ITEMS

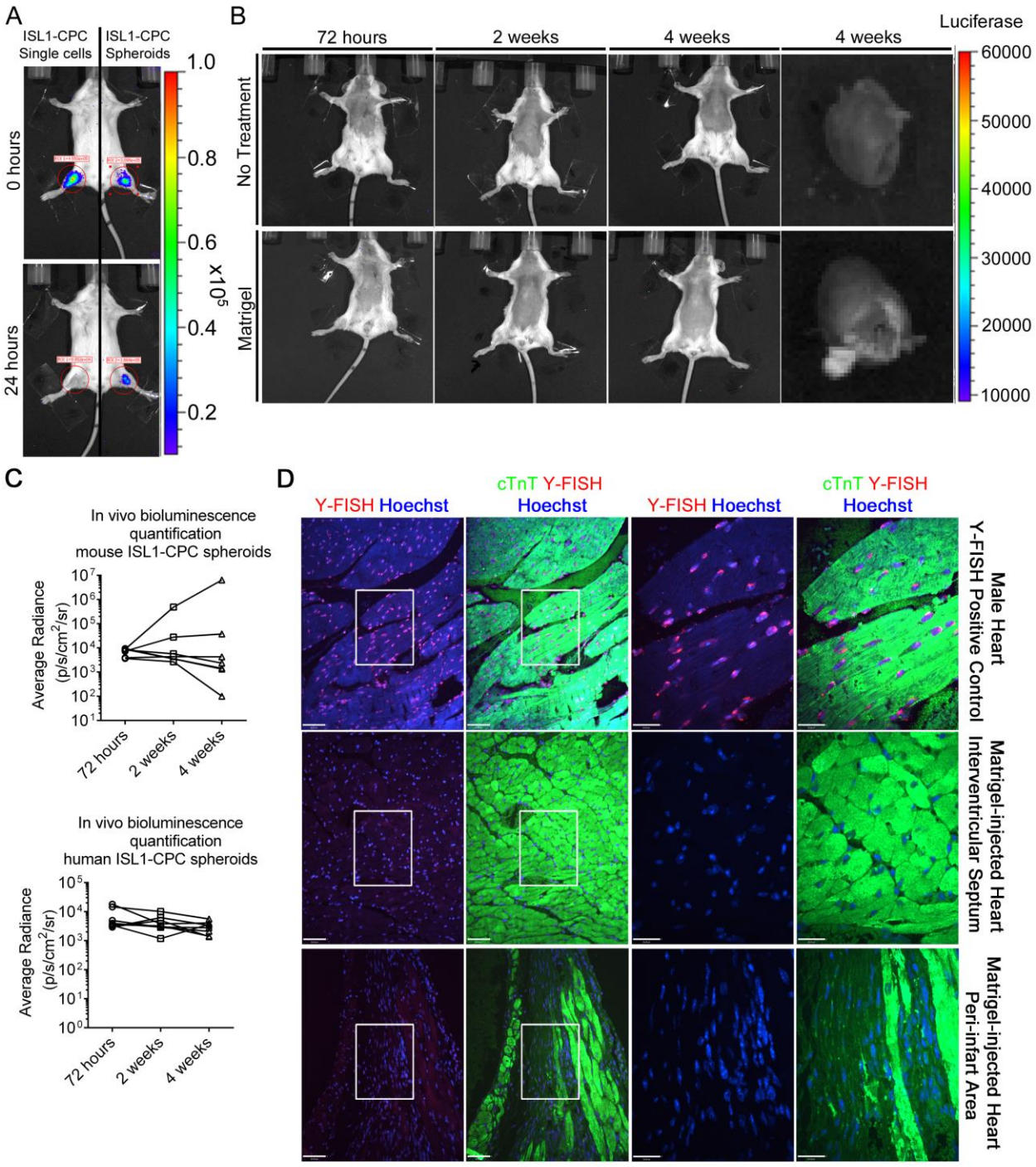
Supplemental Figure 1. Related to Figure 1



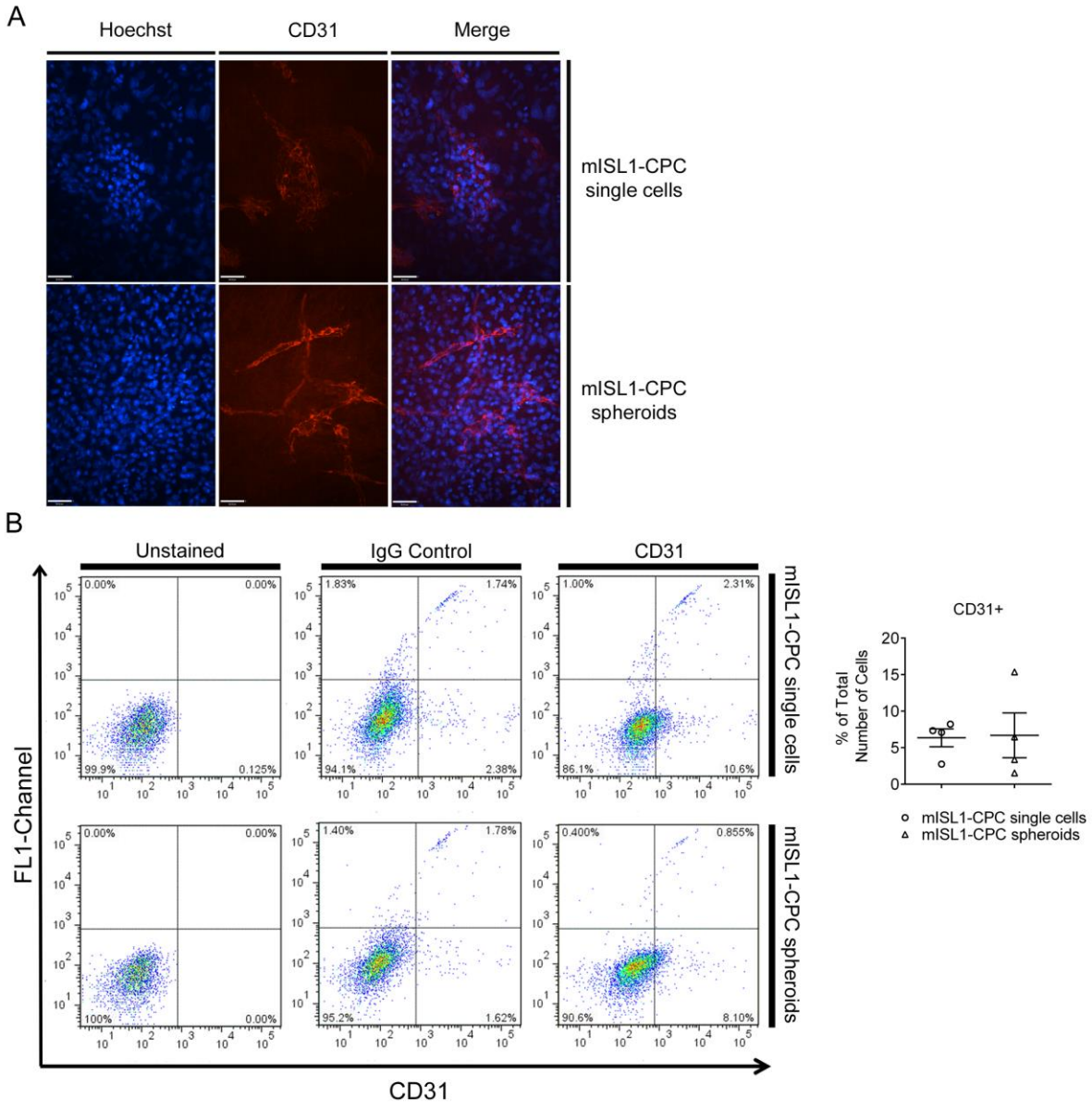
Supplemental Figure 2. Related to Figure 1



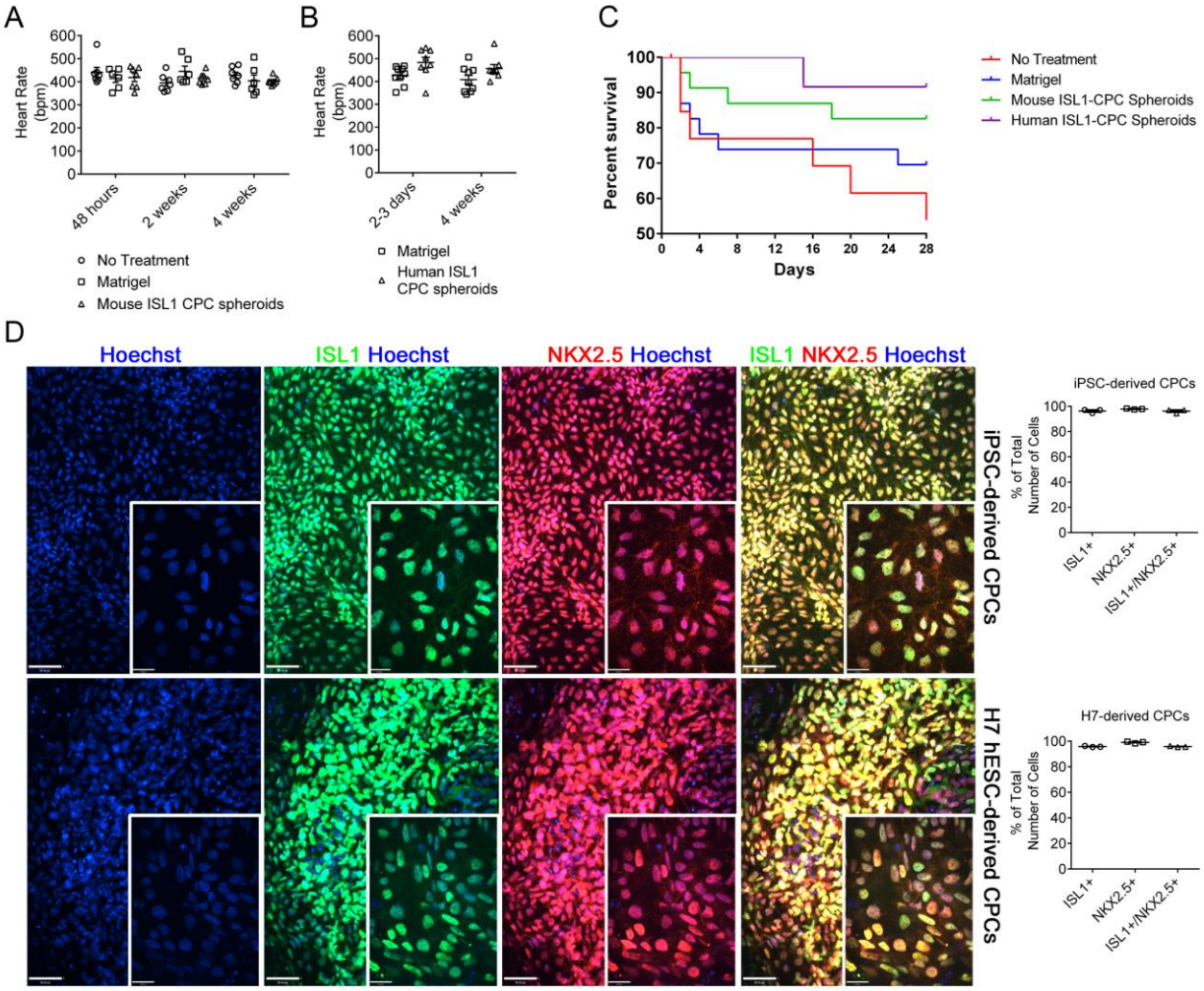
Supplemental Figure 3. Related to Figure 2



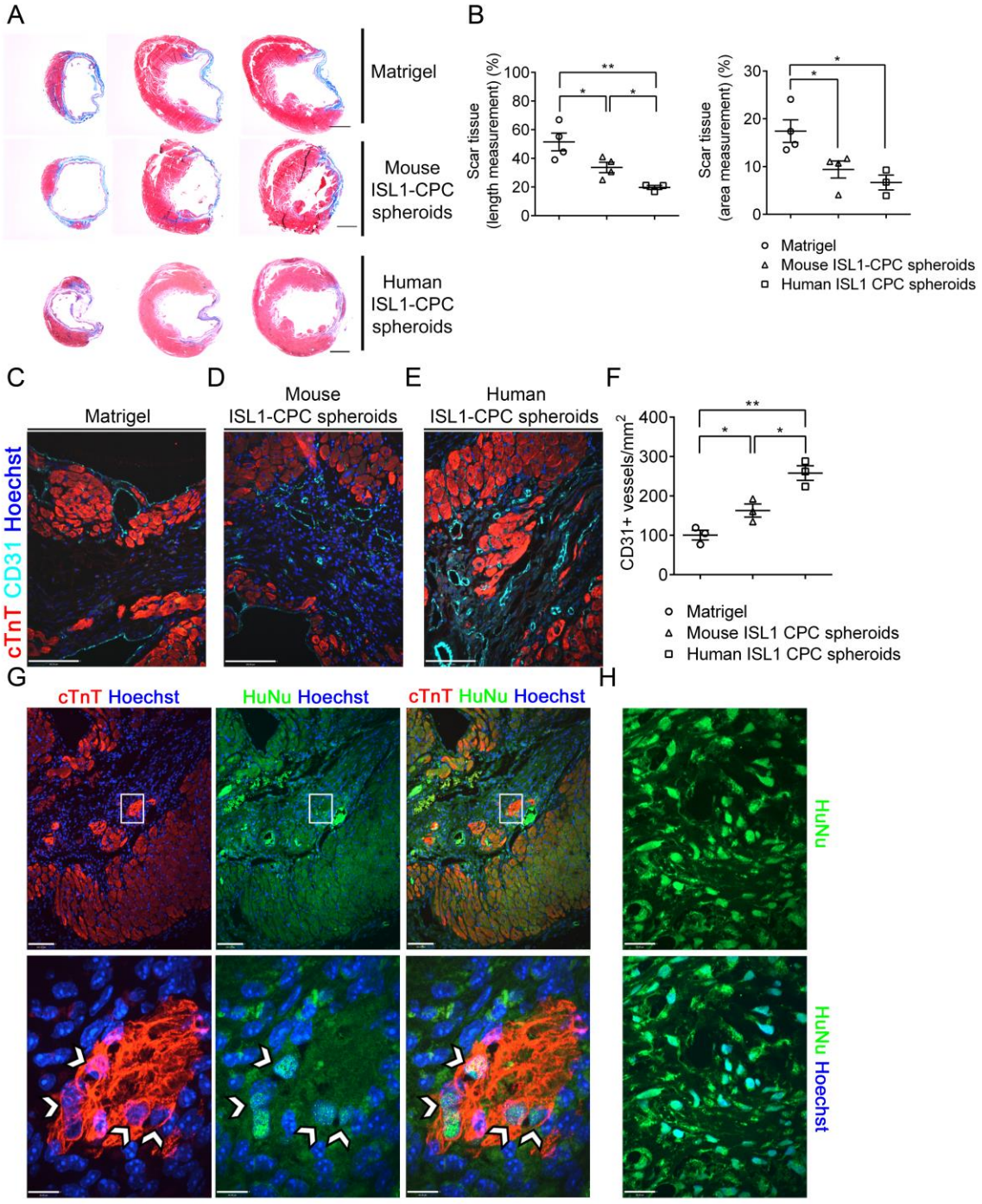
Supplemental Figure 4. Related to Figure 2



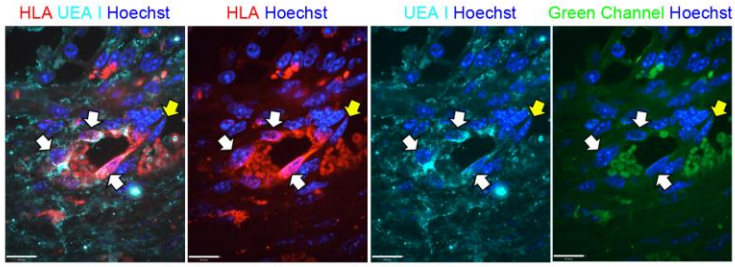
Supplemental Figure 5. Related to Figures 3, 4 and 8



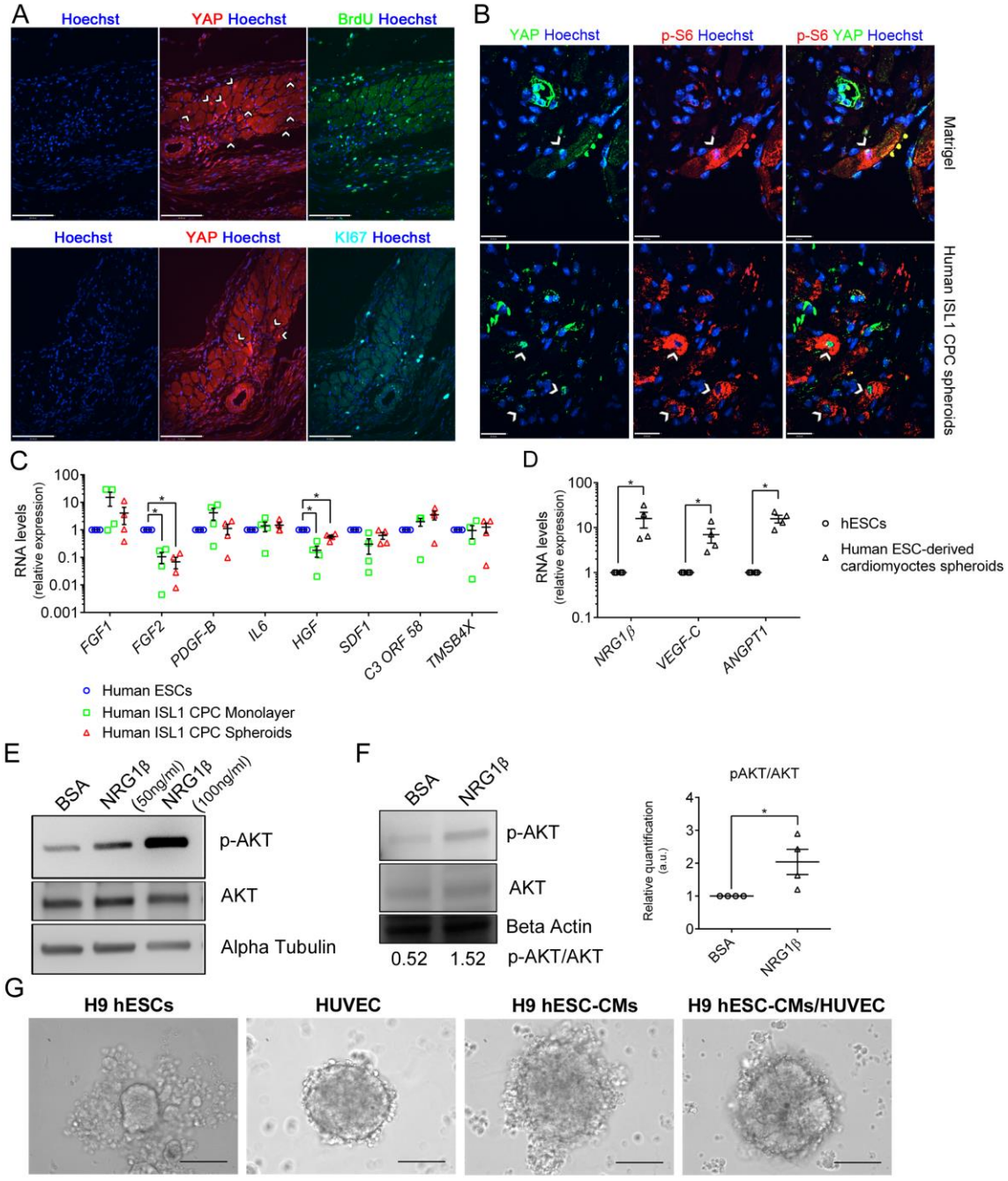
Supplemental Figure 6. Related to Figures 2 and 8



Supplemental Figure 7. Related to Figure 8



Supplemental Figure 8. Related to Figures 9 and 10



Supplemental Figure 1. Generation of double transgenic mESC line for isolation of ISL1-CPCs.
A. Schematic representation of the transgenes contained in the double transgenic mESC line AHF-GFP/p-Ub-fluc-mRFP-tTk. **B.** Isolation of ISL1-CPCs with the double transgenic mESC line. EB5.5 pictures represent bright field (left), monomeric RFP (middle) and GFP (right) detection. Scale bar, 100µm. Representative FACS sorting plot showing the percentage of GFP+ cells on EB5.5. **C.** Fixed mISL1-CPC spheroids showed neither green nor red fluorescence. Therefore, neither GFP nor RFP interfered with immunofluorescence staining performed *in vitro* or in histological sections. Scale bar, 70µm.

Supplemental Figure 2. ISL1-CPC spheroid formation under small molecule treatment.
A. ISL1-CPC spheroid treatment for 24 hours with DMSO (left), 10µM ICG001 (middle) and 10µM TNP470 (right). Scale bar, 100µm. At the bottom row, LIVE/DEAD (green/red) staining in ISL1-CPCs plated on fibronectin-coated plates and treated for 24 hours with DMSO (left), 10µM ICG001 (middle) and 10µM TNP470 (right). Green signal represents calcein staining for live cells; Red signal represents ethidium homodimer staining for dead cells. Scale bar, 100µm. **B**. Time-course experiment for ISL1-CPC spheroid formation in control conditions or in the presence of 5µM Y-27632 (Rock kinase inhibitor). Scale bar, 100µm.

Supplemental Figure 3. Bioluminescence imaging in hindlimbs and control treatment groups.
A. Mouse ISL1-CPC single cells (left side of each image) or mISL1-CPC spheroids (right side of each image) were injected in the hindlimbs of SCID-bg females. Imaging was recorded 10 minutes (top panels) or 24 hours (bottom panels) after cell injection. ISL1-CPC single cells were undetectable 24 hours after injection. Representative animal from n=4. **B.** Bioluminescent images in a representative live animal without treatment (top row) or injected with Matrigel (bottom row) after left coronary artery ligation and recorded at three different time points: left column (72 hours); middle column (2 weeks); right column (4 weeks). Hearts extracted 4 weeks after surgery/treatment are presented on the far right column. In A and B, the numbers in the scale bars were introduced manually to enlarge the font size. **C.** Graphs representing the bioluminescence signal as average radiance, quantified in live animals injected with mISL1-CPC spheroids, n=7 (top graph) or hISL1-CPC spheroids, n=8 (bottom graph). Statistical analysis performed with one-way repeated measurements ANOVA with Greenhouse-Geisser correction (mISL1 CPC spheroids, p=0.35; hISL1-CPC spheroids, p=0.12)**D.** Y-FISH staining in a heart section of a male mouse (top panels) and a section of a Matrigel-injected female mouse (middle and bottom panels). Matrigel-injected heart was extracted 4 weeks after left anterior coronary artery ligation. cTnT staining in green and Y-FISH staining in red. Note the staining for Y-FISH in the male heart and the absence of specific staining for Y-FISH in the female heart injected with Matrigel. Images in the first and second columns from left show 20x and those from the third and fourth columns from left represent 60x magnifications. White rectangles in 20x pictures represent the regions magnified in 60x. Scale bars, 50µm for 20x and 20µm for 60x.

Supplemental Figure 4. Endothelial cell differentiation of mouse ISL1-CPCs (mISL1-CPCs).

A. Staining for CD31 (red) in mISL1-CPCs plated for 1 week on reduced growth factor-Matrigel-coated plates. CD31+ cells are detected in mISL1-CPC single cells (top) and mISL1-CPC spheroid (bottom) differentiated cells. Scale bar, 50µm. **B.** One week after plating on Matrigel, mISL1-CPC single cells (top) or spheroids (bottom) were subjected to FACS analysis to quantify the percentage of CD31+ cells. No significant differences were detected between single cells and spheroids. n=4 independent experiments. Statistical analysis by two-tailed Student t-test.

Supplemental Figure 5. Heart rates and animal survival in infarcted animals and ISL1 CPCs derived from iPSCs and H7 hESCs.
A. Heart rates of the animals used in the study at 3 different time points were comparable. n=7 for no treatment and mISL1-CPC spheroids groups and n=6 for Matrigel group. Samples were analyzed by two-way ANOVA. **B.** Heart rates obtained from B-mode, parasternal long axis, measured in the mid-part of the left ventricle. Statistical analysis performed by two-tailed Student t-test. n=8 for Matrigel and hISL1 CPC spheroids. **C.** Kaplan-Meier survival curves for animals undergone myocardial infarction and different treatments. Mantel-Cox test: hISL1-CPC spheroids vs. Matrigel, p-value= 0.14 ; hISL1-CPC spheroids vs. no treatment, p-value= 0.04; mISL1-CPC spheroids vs. Matrigel, p-value= 0.29; mISL1-CPC spheroids vs. no treatment, p-value= 0.07; Matrigel vs. no treatment, p-value= 0.40, n=14 for no treatment, n=23 for Matrigel and mISL1-CPC, and n=14 for hISL1 CPC groups. **D.** ISL1 and NKX2.5 stainings in CPCs from induced pluripotent stem cell (iPSCs) derived from a healthy patient (top panels) or H7 hESCs (bottom panels). Scale bars, 50µm in 20x pictures and 20µm in 60x insets. Graphs showing quantification of n=3 independent experiments. Quantifications are presented as mean±SEM. In iPSC-derived CPCs: ISL1+ (96.29±0.81); NKX2.5+ (97.91±0.20); ISL1+/NKX2.5+ (96.20±0.84). In H7-derived CPCs: ISL1+ (95.79±0.26); NKX2.5+ (99.03±0.41); ISL1+/NKX2.5+ (95.79±0.26).

Supplemental Figure 6. ISL1-CPC spheroids decrease scar tissue and enhance blood vessel formation.
A. Masson's Trichrome staining showing 3 coronal sections of a representative mouse injected with Matrigel (top row), mouse ISL1-CPC spheroids (middle row) or human ISL1-CPC spheroids (bottom row). Scale bar, 1mm. **B.** Quantification of scar tissue based on Trichrome staining, using two different methods: 1) length measurements; 2) area measurements. n=4 for Matrigel and mouse ISL1-CPC spheroids and n=3 for human ISL1-CPC spheroids. Measurements were performed in every heart at 3 different points that were matched between experimental groups. **Length measurements: (**Endocardial ratio+ Epicardial ratio)/2 x 100. **Endocardial/Epicardial ratio:** (Endocardial or Epicardial Scar Length/Endocardial or Epicardial Circumference).
For scar area measurements, areas were delimited manually and quantified automatically with ImageJ. Statistical analysis performed by two-tailed unpaired Student t-test. *$p<0.05$; **$p<0.01$. **C-F.** Representative images of a heart injected with Matrigel (C), mouse ISL1-CPC spheroids (D) or human ISL1-CPC spheroids (E) 4 weeks after surgery/ treatment. Blood vessels were stained with a CD31 antibody (cyan). Cardiac muscle (cTnT) is shown in red. Scale bar, 100µm. **F.** Graph representing blood vessel quantification in the scar area. Three matched slides were quantified per heart. Graph represents the average of n=3 hearts per experimental condition.

Statistical analysis performed by two-tailed unpaired Student t-test. *p<0.05; **p<0.01. **G.** Human ISL1-CPC spheroids differentiated to cardiomyocytes in infarcted hearts, 4 weeks after injection, as shown by human nuclear (HuNu) antigen staining. Bottom panels show higher magnification of the area demarcated by the white rectangle in the top panels. White arrowheads show cTNT+/HuNu+ cells. Scale bars, 100µm and 20µm for top and bottom panels respectively. **H.** Paraffin embedded iPSC-derived smooth muscle cells used as positive control for HuNu staining. Scale bar, 20µm.

Supplemental Figure 7. Human ISL1-CPC spheroid-derived cells form functional blood vessels in infarcted mice.
Human ISL1-CPC-derived endothelial cells (ECs) were detected by co-immunostaining of HLA (red) and biotinilated *Ulex europaeus* aglutinin I (UEA I, cyan) (white arrows), a lectin that binds glycoproteins and glycolipids in human ECs, 4 weeks after cell implantation. Note the presence of a mouse blood vessel, negative for HLA and UEA I (labeled with a yellow arrow). The green channel revealed the presence of autofluorescent red blood cells, indicating that human ISL1-CPC-derived ECs were functional and lined the blood vessels. Scale bar, 20µm.

Supplemental Figure 8. Human ISL1-CPC spheroids in infarcted hearts and spheroid generation with different human cell types.
A. Staining for YAP and BrdU (top panels) or YAP and KI67 (bottom panels) in an infarcted heart injected with hISL1-CPC spheroids, 4 weeks after surgery and cell delivery. Arrowheads show nuclear YAP in host cardiomyocytes. Scale bars, 100µm. **B.** Host cardiomyocytes with active YAP (green) expressed a high level of the phosphorylated ribosomal subunit S6 (p-S6) (red). White arrowheads show host cardiomyocytes with active (nuclear) YAP. Scale bar 20µm. **C.** Growth factor and cytokine gene expression profile in hISL1-CPC spheroids. Genes were selected based on their cardiac protection effects reported in the literature. n=4 independent experiments. Statistical analysis performed with Mann-Whitney U test. *p<0.05. **D.** Growth factor gene expression profile in day 15 H9 ESC-derived cardiomyocyte spheroids. n=4 independent experiments. Statistical analysis performed with Mann-Whitney U test. *p<0.05. **E.** Representative western blot (n=2) showing a dose-response effect of NRG1β in AKT activation in iPSC-derived cardiomyocytes under serum and glucose deprivation. **F.** Western blots and graph showing that NRG1β (100 ng/ml) induced AKT activation in human iPSC-derived cardiomyocytes under hypoxia and serum and glucose deprivation. n=4 independent experiments. Statistical analysis performed with Student t-test. *p<0.05. **G.** Methylcellulose-induced spheroids with H9 human embryonic stem cells (hESCs), human umbilical vein endothelial cells (HUVEC), H9 hESC-derived cardiomyocytes (hESC-CMs) and co-spheroids hESC-CMs + HUVEC. Spheroids were prepared for 24 hours with 5µM Y-27632, using 1,000 cells/spheroid. Co-spheroids were prepared with 500 hESC-CMs plus 500 HUVEC per spheroid. Scale bar, 100µm.

MOVIES

Movie 1. Murine ISL1-CPC spheroids on Matrigel.

ISL1-CPC spheroids were formed for 24 hours and then cultured for 6 days on growth factor reduced Matrigel-coated plates.

Movie 2. Murine ISL1-CPC single cells on Matrigel.

ISL1-CPC single cells were plated after FACS sorting and then cultured for 7 days on growth factor reduced Matrigel-coated plates.

Movie 3. Human ISL1-CPC spheroids on Matrigel.

Day 6 human ISL1-CPCs were detached from 8-well chamber slides and spheroids prepared with methylcellulose, in the presence of 5μM Y-27632 for 24 hours. ISL1-CPC spheroids were plated on Matrigel-coated plates for 10 additional days. Beating was detected 3-4 days after plating.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Double transgenic mESC line AHF-GFP/ pUb-fluc-mRFP-tTk.

A single transgenic AHF-GFP mESC line was obtained by isolating cells of the inner cell mass of day 3 embryos from the mouse transgenic line described in *Qyang et al.* (1). The AHF-GFP line allows the isolation of ISL1 cardiovascular progenitor cells (ISL1-CPCs) based on GFP detection. GFP expression is driven by an anterior heart field enhancer contained in *Mef2c* gene (Supp. Fig. 1A). The double transgenic line was generated after insertion of a cassette containing a fusion protein (firefly luciferase, monomeric red fluorescent protein and truncated thymidine kinase) driven by the human ubiquitin promoter (p-Ub-fluc-mRFP-tTk) (Supp. Fig. 1A). This cassette was described in *Cao et al.* (2). pUb-fluc-mRFP-tTk was delivered by lentiviral transduction using multiplicity of infection (MOI) 10. Ten clones were selected based on luciferase activity detection. The clone chosen to perform the study was the one with the highest ISL1-CPC yield based on fluorescent activated cell sorting (FACS) analysis.

Culture of the mESC line.

The double transgenic mESC line was maintained on a feeder layer of irradiated mouse embryonic fibroblast (MEFs). The culture medium was prepared with Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 15% Knock-out serum replacement (KO-SR, Life Technologies), LIF-conditioned media at 1:500 dilution (obtained from Chinese hamster ovary (CHO) cells stably expressing Leukemia Inhibitory Factor gene), 0.1mM non-essential amino acid (MEM-NEAA, Life Technologies), 2mM L-glutamine (Life Technologies), 1mM sodium pyruvate (Life Technologies), 1% penicillin and streptomycin (Life Technologies) and 0.1mM 2-mercaptoethanol (Sigma).

Isolation and culture of mouse ISL1-CPCs.

mESCs were dissociated with 0.05% Trypsin-EDTA (Life Technologies) and cultured in feeder-free conditions to deplete MEFs before differentiation. For feeder-free culture, mESCs were plated on gelatin-coated tissue culture dishes for 2 days. The medium used for feeder-free culture was Iscove's modified Dulbecco's medium (IMDM, Life Technologies) supplemented with 15% KO-SR, LIF-conditioned medium, 2mM L-glutamine, 1% penicillin and streptomycin

and 0.1mM 1-thioglycerol (Sigma). Cells were dissociated after 2 days in feeder-free culture with 0.05% Trypsin-EDTA and suspended in differentiation medium. In order to obtain ISL1-CPCs, mESCs were differentiated following the established hanging drop method. A suspension containing 3.6 X 10^4 cells per ml was prepared for hanging drops, delivering 15µl/ drop (540 cells). 2 days later, hanging drops were collected with differentiation medium and plated on Petri dishes (not treated for cell culture) for additional 3.5 days (embryoid body day 5.5; EB5.5). Differentiation medium contained IMDM, 15 % fetal bovine serum (Gemini), 50 µg/ml ascorbic acid (Sigma), 0.1mM 1-thioglycerol (Sigma) and 2mM L-glutamine. At EB5.5 cells were collected and dissociated first with collagenase A and B (10mg/ml, Roche) and later with accutase (1mg/ml, Life Technologies) to obtain single cells. Cells were filtered with sterile cell strainers (BD Biosciences) before FACS sorting to remove cluster of cells.

Human ISL1-CPCs.

Briefly, single cells were obtained from 85-90% confluent hESCs with 1mg/ml Accutase. $5x10^4$ cells/cm^2 were seeded on Matrigel-coated (1:30) 8-well glass chamber slides (Thermo Scientific) with mTeSR containing 5µM Y-27632 (Rock kinase inhibitor, Calbiochem). The following day the medium was replaced with fresh mTeSR to remove Y-27632. Four days after plating, differentiation was started by replacing mTeSR with B27 minus insulin (Life Technologies) in RPMI 1640 (Life Technologies) (B27/RPMI) containing 12µM CHIR99021 (Selleckchem) (Day 0). Twenty-four hours later, medium was replaced with fresh B27/RPMI. On day 3 of differentiation medium was replaced with B27/RPMI containing 5µM IWP4 (Stemgent). On day 5 medium was again replaced with fresh B27/RPMI. ISL1-CPCs were analyzed on day 6 of differentiation. Similar efficiency for ISL1-CPC has been obtained with the transgene-free human ESC line H7 (WiCell Research Institute) and an iPSC cell line derived from a healthy donor (Yale Stem Cell Center Core).

Human ESC-derived cardiomyocytes.

ESC differentiation to cardiomyocytes was performed as previously described by Lian et al., 2013.

ISL1-CPCs in 96-V wells and hanging drops.

To test murine ISL1-CPC spheroid formation by forced aggregation in 96-V plates (conical bottom, Thermo Scientific), 1,000 cells per well were plated with the corresponding differentiation medium. Cells were pelleted down by centrifugation at 950xg for 5 minutes at room temperature as described (3).

For hanging drops, 1,000 cells per 15µl drop were prepared in the corresponding differentiation medium.

Methylcellulose-induced ISL1-CPC spheroid formation and treatments.

Briefly, a solution containing 1.2% methylcellulose (Sigma) in differentiation medium was prepared by adding 125ml of 60ºC pre-warmed differentiation medium to 3g of autoclaved methylcellulose. Methylcellulose was mixed using magnetic stirring for 20 minutes at room temperature. After this, 125ml of room temperature differentiation medium were added and the

final solution was mixed for 2 hours at 4ºC using magnetic stirring. Debris was removed by centrifugation at 5,000xg for 2 hours at 4ºC. For spheroid formation, FACS-isolated ISL1-CPCs were suspended in 1:5 (1.2% methylcellulose: differentiation medium), obtaining 0.24% final concentration of methylcellulose. Cells suspended in this solution were plated on low attachment-round bottom 96 well plates (Corning). In order to form spheroids containing 1,000 cells/ spheroid, 100µl (10 ISL1-CPCs/ µl) cell suspension were added to each 96-well.

Treatments were performed during the spheroid formation process by mixing the drugs with the ISL1-CPC-containing solution mentioned above. For spheroid formation experiments, different concentrations of drugs were tested during a period of 24 hour. 10µM ICG001 (Calbiochem) and 10µM TNP470 (Sigma) showed strong blocking activity during ISL1-CPC spheroid formation. DMSO was used as control for these drugs. On the other hand, 5µM Y27632 enhanced ISL1-CPC spheroid formation. Note that Y27632 was not included during mISL1-CPC spheroid formation in experiments in which spheroids were injected in hindlimbs or infarcted hearts.

For human ISL1-CPC spheroid formation, cells were isolated on day 6 of differentiation with 1mg/ml Accutase. ISL1-CPC spheroids were prepared with B27 minus insulin in RPMI 1640 containing 20% IMDM, 3% FBS and 0.24% methylcellulose. Treatments used were performed as mentioned above, with 5µM Y27632, 50µM RAC1 inhibitor NSC23766 (Santa Cruz Biotechnologies), 10µM ERK1/2 inhibitor U0126 (Cell Signaling), 10µM P38 inhibitors BIRB 796 and SB203580, or 10µM JNK inhibitor SP600125 (Calbiochem).

Methylcellulose-induced spheroids with other human cell types.

H9 hESCs were maintained in feeder-free with mTeSR and differentiated to cardiomyocytes (CMs) as previously described (4). Human umbilical vein cells (HUVEC) were maintained in EGM medium (Lonza). H9 hESCs, hESC-CMs and HUVEC were detached with 1mg/ml Accutase for 10-15 minutes to obtain a single cell suspension. Spheroids were formed in the presence of 5µM Y-27632 for 24 hours, with 1,000 cells /spheroid. Co-spheroids were prepared by mixing 50% hESC-CMs and 50% HUVEC.

Fluorescent Activated Cell Sorting (FACS) analysis.

ISL1-CPCs were plated on Matrigel-coated plates as described above. 1 week after seeding (7 days for ISL1-CPC single cells and 6 days for ISL1-CPC spheroids) cells were dissociated with collagenase A and B (10mg/ml) and posteriorly with accutase to get single cells. To reduce dead cell noise for FACS analysis, cells were stained with Fixable Viability Dye eFluor 780 (eBiosciences) for 30 minutes at 4ºC. Unlabeled dye was removed with several phosphate buffer saline (PBS) washes and cells were fixed with 2% paraformaldehyde (PFA) for 10 minutes at room temperature. Blocking was performed with 10% normal goat serum (NGS) in PBST (0.1% Triton in PBS) at room temperature for 1 hour. Cells were incubated with 1µg/ml of mouse anti cardiac troponin T (cTnT) (Thermo Scientific) and 1µg/ml rabbit anti smooth muscle alpha actin (SMA) (Abcam) or 1µg/ml rabbit anti CD31 (Abcam) in blocking solution overnight at 4ºC. Samples with IgGs control were incubated with 1µg/ml mouse IgG1 (Thermo Scientific) and 1µg/ml normal rabbit IgG (Santa Cruz). Secondary antibodies goat anti mouse Alexa Fluor 488 and goat anti rabbit Alexa Fluor 568 (Life Technologies) were incubated in blocking

solution 1 hour at room temperature. Samples were analyzed using an LSRII machine (BD Biosciences) and FlowJo software. Dead cells were gated using APC-780 and discarded from the analysis and only singlets were considered for final quantification (FSC-H vs FSC-A).

Immunofluorescence.

Cells were fixed with 4%PFA for 10 minutes at room temperature. When heart sections were used for staining, samples were deparaffinized with xylene, rehydrated and subjected to antigen retrieval with 10mM sodium citrate at 90ºC for 20 minutes. Samples were blocked with blocking solution (10% NGS in PBST) for 1 hour at room temperature and incubated with mouse anti-cTnT (1:250, Thermo Scientific #MS-295), rabbit anti-cTnT (1:250, Abcam #ab92546) rabbit anti-SMA (1:200, Abcam #ab5694), rabbit anti-CD31 (1:100, Abcam #ab28364), mouse anti-Isl1 (1:100, clone 39.4D5, Developmental Studies Hybridoma Bank), rabbit anti-Nkx2.5 (1:50, Santa Cruz Biotechnology #sc-14033), mouse anti-active β-catenin (clone 8E7, 1:100, Millipore #05-665), rabbit anti-human leukocyte antigen (1:50, Abcam #ab52922), mouse anti-YAP (1:50, Santa Cruz Biotechnologies #sc-101199), rabbit anti-phospho-S6 (Ser240/244, 1:100, Cell Signaling #D68F8), mouse anti-human nuclear antigen (1:20, Millipore #MAB1281), rabbit anti-Ki67 (1:100, Abcam #ab16667), rat anti-BrdU (1:100, Abcam #ab6326), rabbit anti-N cadherin (1:50, Santa Cruz Biotechnologies #sc-14033), mouse anti-RAC1 (1:100, BD Transduction Laboratories #610650), rabbit anti-IQGAP1 (1:50, Santa Cruz Biotechnologies #sc-10792) in blocking solution at 4ºC overnight. Secondary antibodies were incubated in blocking solution 1 hour at room temperature. To detect human ISL1-CPC-derived endothelial cells, biotinylated *Ulex europaeus* aglutinin I (20µg/ml, Vector Laboratories #B-1065) was incubated for 1 hour at room temperature, following manufacturer's instructions. Ulex was detected with an A647-conjugated monoclonal mouse anti-biotin antibody (1:100, clone 3D6.6, Jackson ImmunoResearch #200-602-211). Nuclei were stained with Bisbenzimide H 33258 dye (Hoechst, 1:1000, Sigma).

Heart isolation.

Mice were anesthetized with intraperitoneal injection of 100mg/Kg ketamine and 10mg/Kg xylazine. Once no noxious response was detected to a stimulus, the thoracic cavity was opened. Right atrium was cut to help blood removal from the chambers and heart was arrested in diastole after injection of 0.5ml of 1M KCl in the left ventricle. Blood was removed from the heart after injection of 10ml PBS. Once the heart was cleared up, it was extracted from the animal and imaged in a culture plate containing 150µg/ ml luciferin (Gold Biotechnology). Images were recorded with an IVIS200 device (Caliper, PerkinElmer). Hearts were fixed with formalin overnight at room temperature and processed for paraffin embedding.

Neonatal rat cardiomyocytes isolation, culture and treatments.

Neonatal rat cardiomyocytes were isolated from 1 day old Sprague Dawley rats (Charles River Laboratories) using the Primary Cardiomyocyte Isolation Kit from Thermo Fisher Scientific, according to manufacturer recommendations. Cardiomyocytes were plated on fibronectin-coated plates and maintained in DMEM for primary cells containing 10% FBS, 2% horse serum, penicillin/streptomycin and growth factors included in the kit. Experiments were performed 4-5 days after cell plating. In all the experiments, serum was removed 24 hours in

advance. For YAP activation experiments with NRG1β or human ISL1-CPC spheroids conditioned medium, cells were pre-incubated for 2-3 hours in DMEM without glucose. Bovine serum albumin (BSA) or 100ng/ml of recombinant human NRG1β (R&D Systems #396-HB) were added to the cells in glucose-free DMEM. Cells were incubated under hypoxia (1% O2, 5% CO2, 94% N2) for 30 minutes and fixed for immunofluorescence staining. In experiments with conditioned medium, human ISL1-CPC spheroids were plated on Matrigel-coated plates for 24 hours in the presence of B27 (minus insulin)/RPMI 1640. Lapatinib (Selleck Chemicals #S1028) or DMSO was added to neonatal rat cardiomyocytes 30 minutes before adding the conditioned medium. Conditioned medium was filtered and added to rat cardiomyocytes in the presence of DMSO or 2μM Lapatinib. B27 (minus insulin)/RPMI 1640-containing DMSO was used as control conditioned medium. Rat cardiomyocytes were incubated for 2 hours under hypoxia. For AKT activation experiments, BSA or 100ng/ml NRG1β were added to neonatal rat cardiomyocytes in glucose-free DMEM and cells were incubated for 12 hours under hypoxia.

Fibrosis in adult human ventricular cardiac fibroblasts.

Adult ventricular cardiac fibroblasts were purchased from Sciencell Research Laboratories (#6310) and cultured in high glucose DMEM with 10% FBS. For TGFβ1-induced fibrosis experiments, fibroblasts were grown on serum-free conditions 24 hours before and during the induction of fibrosis. Fibrosis was induced with 15ng/ml recombinant human TGFβ1 (Peprotech #100-21C). NRG1β, VEGF-C (R&D Systems #2179-VC) or ANGPT1 (R&D Systems #923-AN) were added at 100ng/ml together with TGFβ1. All treatments were performed for 48 hours. Fibroblasts without any treatment, but grown on serum-free medium were used as control.

RAC1 pull-down experiment.

RAC1 pull-down experiment was performed using a kit from Cytoskeleton Inc (#BK035). Briefly, day 6 human ISL1-CPCs were detached with Accutase and suspended in 0.24% methylcellulose for 45 minutes with or without 5μM Y27632. RAC1-GTP was pulled-down with 10μl PBD-PAK beads, using 400μg of protein.

Western blot.

Protein was extracted with RIPA buffer containing protease and phosphatase inhibitors and samples were loaded in 4-15% Tris-Glycine gels (BioRad) in denaturing conditions with SDS buffer. Blots were incubated with mouse anti-YAP (1:200), rabbit anti-phospho-YAP (Ser127, 1:1000, Cell Signaling #4911), rabbit anti-phospho-AKT (Ser473, 1:1000, Cell Signaling #4058), mouse anti-pan AKT (40D4, 1:1000, Cell Signaling #2920), mouse anti-collagen 1a1 (1:1000, Santa Cruz Biotechnologies #sc-28657), rabbit anti-SMA (1:1000), mouse anti-RAC1 (1:1000), mouse anti-α tubulin (B-5-1-2, 1:1000, Sigma Aldrich #T5168) and mouse anti-β actin (AC-15, 1:1000, Sigma Aldrich #A5441). Western blot quantifications were performed with Image J.

Real time-reverse transcription PCR (RT-qPCR)

RNA was extracted with Trizol (Life Technologies), reverse transcriptions (RTs) were performed with iScript Reverse Transcription Supermix (BioRad) and qPCRs with iQ SYBR Green SuperMix (BioRad) using a BioRad CFX96 qPCR System. Data presented was obtained with $2^{-\Delta\Delta CT}$ method.

Primers (presented 5'-3' orientation):

NRG1β Forward: CTTCTTCATCTACATCTACATC

NRG1β Reverse: CAAGATGCTTGTAGAAGCTG

VEGF-C Forward: GATGCTGGAGATGACTCAAC

VEGF-C Reverse: TACAGACACACTGGCATGAG

ANGPT1 Forward: GGTACTAAATCAAACTTCTCG

ANGPT1 Reverse: CTTCCTTGTGTTTTCCTTCC

FGF1 Forward: GCACATTCAGCTGCAGCTC

FGF1 Reverse: CTTCTCTGCATGCTTCTTGG

FGF2 Forward: TTCTTCCTGCGCATCCACC

FGF2 Reverse: CGTAACACATTTAGAAGCCAG

PDGF-B Forward: CCTGTCTCTCTGCTGCTAC

PDGF-B Reverse: GTCATGTTCAGGTCCAACTC

IL6 Forward: AGAAAGGAGACATGTAACAAG

IL6 Reverse: CCTCACTACTCTCAAATCTG

HGF Forward: CATTCACTTGCAAGGCTTTTG

HGF Reverse: TCTTAGTGATAGATACTGTTCC

SDF1 Forward*:* TGAAGAACAACAACAGACAAG

SDF1 Reverse: GCAAAACAAAGCCCTTGGC

C3 ORF 58 Forward: TGTTTTGGTTGCTGACAAAAG

C3 ORF 58 Reverse: GGATAAGAGGTTCTGACAAAC

TMSB4X Forward: AAAGAAACGATTGAACAGGAG

TMSB4X Reverse: TGTCAGTAGTTCTTTGATGTG

E-Cadherin Forward: GCAAGGTTTTCTACAGCATC

E-Cadherin Reverse: AGAGAAGAGAGTGTATGTGG

N-Cadherin Forward: TATGCCGTGAGAAGCTTTCC

N-Cadherin Reverse: GTGCTTACTGAATTGTCTTGG

VE-Cadherin Forward: CGATAATTCTGGACGTATTATC

VE-Cadherin Reverse: TGTACTTGGTCTGGGTGAAG

DSG1 Forward: TCCTTTCTTCATTATCTACTGC

DSG1 Reverse: CATTGAGTATCATCACCAGTG

GAPDH Forward: GAAGGTGAAGGTCGGAGTCA

GAPDH Reverse: TTGAGGTCAATGAAGGGGTC

Y-chromosome FISH staining.

Y-probe was produced as described previously (5). For details on performing FISH staining on paraffin sections, see *Theise et al.* (6).

Masson's Trichrome staining.

Heart coronal sections were deparaffinized and rehydrated. Staining was performed with Masson's Trichrome Stain kit, Artisan (Dako). In brief, sections were re-fixed with Bouin's solution for 1 hour at 56ºC. After rinsing with tap water, they were stained with Weigert's iron hematoxilin for 10 minutes, rinsed with water and stained with Biebrich scarlet-acid fuchsin solution for 15 minutes. Samples were rinsed with water and incubated with phosphomolybdic-phosphotungstic acid until red staining was removed from the scar area. Collagen was stained with aniline blue. Three matched regions were considered per heart for scar area quantification, following the length and areabased measurements described (7). Length measurements: (endocardial ratio+ epicardial ratio)/2 x 100. Endocardial or epicardial ratio was calculated as endocardial or epicardial scar length normalized by endocardial or epicardial circumference, respectively. For scar area measurements, left ventricular areas were manually delimited and automatically quantified using ImageJ. Area measurements: sum of scar areas/sum total surface areas x 100.

LIVE/DEAD staining.

LIVE/DEAD staining was performed on mouse ISL1-CPCs seeded for 24 hours on plates coated with fibronectin (Sigma) following manufacturer's instructions. The treatments tested were DMSO (1:1,000, control), ICG001 and TNP470 (10μM final concentration for both drugs).

For human, ISL1-CPCs were isolated on day 6 as described above and $1x10^5$ cells (single cells) or 100 spheroids ($1x10^5$ cells) were seeded on 24-well Matrigel-coated plates. LIVE/DEAD staining was performed 24, 48 and 72 hours after cell seeding. Only ISL1-CPC

single cells were seeded in the presence of 5μM Y-27632, which was removed 24 hours after plating.

Experimental animals: surgeries and experimental treatments.

Mice were anesthetized with inhaled 2-2.5% isoflurane (v/v) in a closed chamber. After securing them in supine position, a mid-line incision in the neck was performed to facilitate intubation. Animals were intubated with a 21G angiocatheter attached to a rodent ventilator (Harvard Instruments). Controlled respirations of 1.5-2% isoflurane/O_2 (v/v) blend were delivered. Hair was removed with Nair lotion (Church & Dwight Co). Skin was cleaned with betadine and 70% ethanol. A left thoracotomy incision was made at the level of the 4-5 intercostal space followed by retraction of pectoralis muscles. Intercostal space muscles were incised and ribs retracted to get access to the thoracic cavity. Lungs were gently moved supralaterally using Q-tips to uncover the heart. Left coronary artery ligation was performed with 8-0 sterile nylon suture (Aros Surgical). Right after ligation, reduced growth factor-Matrigel or mouse ISL1-CPC spheroids (500 per animal, 1,000 cells/ spheroid) or human ISL1-CPC spheroids (2,000 per animal, 1,000 cells/ spheroid) suspended in 20μl of growth factor reduced Matrigel were administered by a single injection in the peri-infarct area using insulin syringes with a 45-degree angle-31G needle. Non-treated animals did not receive any injection. Ribs and skin were sutured with 6-0 prolene suture (Ethycon). Surgical procedures were performed with water-circulating heating pads (Gaymar) under a Leika MZ6 dissecting microscope. Surgeon was completely blind to treatments administered. Animals were kept for 24-48 hours after surgery with water-circulating heating pads (HTP-1500 Heat Therapy pump, Kent Scientific). Mice without impaired left ventricular anterior wall movement or with normal apex function, analyzed by echocardiography at 48 hours, were removed from the study. Animals that did not contain detectable cells in the heart (bioluminescence imaging) 4 weeks after surgery due to suboptimal cell injection were not considered for the study.

For bromo-deoxy-uridine (BrdU, Sigma Aldrich) administration, 100mg/Kg were injected daily intraperitoneally, starting 2 days after surgery and cell delivery, until the end of the experiments (4 weeks).

Echocardiography.

Ultrasound images were recorded using a Vevo 2100 System (Visual Sonics) at 48 hours, 2 weeks and 4 weeks after surgery. Animals were anesthetized with inhaled 2-2.5% isoflurane/ O_2 (v/v) in a chamber. During imaging recording, 1-1.5% isoflurane/O_2 (v/v) was provided. Animals were kept in supine position on 37ºC heating platforms. Images were recorded along the short axis and parasternal long axis in both cases in the middle part of the left ventricle. Images were analyzed with Vevo 2100 version 1.3.0 software (Visual Sonics). Ejection fraction, end diastolic volume, stroke volume and cardiac output were obtained from B-Mode parasternal long axis measurements. Fractional shortening measurements were obtained from M-Mode short axis. Ultrasound images were recorded and analyzed by 2 experienced technicians with expertise in human and mouse ultrasound imaging. Both technicians were completely blind to the treatments administered in this study.

ISL1-CPC single cells versus spheroid engraftment in hindlimbs.

$1x10^5$ ISL1-CPCs or 100 ISL1-CPC spheroids (approximately $1x10^5$ cells) suspended in phosphate buffer saline were injected per animal in the hindlimbs of SCID-bg females. Bioluminescence imaging was recorded 10 minutes and 24 hours after injection.

SUPPLEMENTAL REFERENCES

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Movie 1. Murine ISL1-CPC spheroids on Matrigel.

ISL1-CPC spheroids were formed for 24 hours and then cultured for 6 days on growth factor–reduced Matrigel-coated plates.

Movie 2. Murine ISL1-CPC single cells on Matrigel.

ISL1-CPC single cells were plated after FACS sorting and then cultured for 7 days on growth factor–reduced Matrigel-coated plates.

Movie 3. Human ISL1-CPC spheroids on Matrigel.

Day 6 human ISL1-CPCs were detached from 8-well chamber slides and spheroids prepared with methylcellulose, in the presence of 5 μM Y-27632 for 24 hours. ISL1-CPC spheroids were plated on Matrigel-coated plates for 10 additional days. Beating was detected 3–4 days after plating.