Online Methods

Preparation of hESC-derived epicardial cells and hESC-derived cardiomyocytes. Epicardial cells were differentiated from GFP-transgenic hESCs as previously described[1]. Briefly, hESCs (H9, WiCell, Madison) were maintained in a chemically defined medium (CDM-BSA) containing Activin-A (10 ng/ml, R&D Systems) and FGF2 (12 ng/ml, R&D Systems). Chemically defined medium consisted of IMDM (250 ml, Life Technologies), Ham's F12 (250 ml, Life Technologies), Pen/Strep (5 ml, Life Technologies), Insulin (350 µl, Roche), Transferrin (250 µl, Roche), chemically defined 100x lipid concentrate (5 ml, Life Technologies) and monothioglycerol (20 µl, Sigma). Differentiation to lateral mesoderm was performed as previously described in CDM-PVA, containing polyvinyl alcohol (PVA, 1 mg/ml, Sigma)[2]. In brief, early mesoderm differentiation was started with a combination of CDM-PVA, FGF2 (20 ng/ml), LY294002 (10 µM, Sigma) and BMP4 (10 ng/ml, R&D) for 1.5 days. Then, lateral mesoderm differentiation was started in CDM-PVA, FGF2 (20 ng/ml) and BMP4 (50 ng/ml) for 3.5 days. To induce epicardial differentiation, cells were resuspended as single cells in CDM-PVA, WNT3A (25ng/ml, R&D), BMP4 (50ng/ml) and RA (4µM, Sigma) at a seeding density of $2.5x10^4/cm^2$ for 10 days and the medium was changed half way through the differentiation. To generate epicardium-derived fibroblasts, epicardial cells were re-plated as single cells at a seeding density of $2.5x10^4/cm^2$ and were grown under the influence of VEGF-B (50 ng/ml, Peprotech) and FGF2 (50 ng/ml) for 12 days. Flow cytometry was performed on day 10 epicardial cells using TCF21 (Atlas antibodies, HPA013189) and WT1 (Abcam, Ab89910) antibodies on BD FACS Calibur (BD Bioscience) analysed using FlowJo VX software version 9.9.4 (Fig. 1, panel b, Supplementary Fig. 8 and 19).

For derivation of mesenchymal stem cells from hESCs, colonies were passaged, resuspended in CDM-PVA containing FGF2 (12 ng/ml) and SB (10µM) and seeded at a density of 30 colonies/cm^2 on gel-MEF coated plates. Cells were enzymatically dispersed and passaged 4 times in CDM-PVA, containing FGF and SB before being split one more time in DMEM-F12 containing 10% fetal bovine serum for long-term maintenance. Primary mesenchymal stem cells (gift from Osiris) were also maintained in DMEM-F12 containing 10% fetal bovine serum.

Cardiomyocytes were generated from hESCs with the ABCX method as previously described[3, 4]. In brief, hESCs (RUES2, Female line, Rockefeller University, NIH registry number 0013) were maintained in feeder-free irradiated mouse embryonic fibroblast (iMEF)-conditioned media containing bFGF (4ng/ml, Peprotech). Cells were seeded as single cells ($1x10^5/cm^2$) on matrigel (BD) coated plates with conditioned media including Chiron 99021 (1μM, Cayman Chemical) and ROCK inhibitor (Y-27632). The following day (day 0), the media was aspirated and cells were fed with RPMI media supplemented with B27 (Invitrogen) containing Activin A (100ng/ml) for 18 hours. On day 1, media was aspirated and cells were fed with RPMI media plus B27 containing BMP4 (5ng/ml) and Chiron 99021 (1μM) for 48 hours. On day 3, media was aspirated and replaced with RPMI media plus B27 containing Xav 939 (1μM, Torcis). On day 5, the medium was replaced with RPMI media plus B27. On day 7, the media was replaced with RPMI containing B27 with insulin (Invitrogen) and was consequently replaced every other day until termination of the protocol.

Cardiomyocytes were frozen down on day 21 and the same batch was used for the entirety of the study. Flow cytometry was performed on thawed cells using cTnT antibody (Thermo, MS-295-P) on BD FACSCanto II (Beckton Dickinson, San Jose, CA) and analysed using FACSDiva software (BD Biosciences), revealing a purity of 97.1%±0.5 (cTnT+, Fig. 1, panel b).

Epicardial cells were heat-shocked on the day prior to cell transplantation, cardiomyocytes prior to freezing, both for 30 minutes at 42.5°. On the day of cell transplantation, epicardial cells and cardiomyocytes were enzymatically dispersed, counted and resuspended in 100μl volume per rat of matrigel and prosurvival cocktail (PSC). PSC consisted of 50% (vol/vol) Matrigel and ZVAD-FMK (100μM, Calbiochem), Bcl-XL (50nM, Calbiochem), Cyclosporin A (200 nM, Wako Pure Chemicals), Pinacidil (50μ, Sigma) and IGF-1 (100ng/ml, Peprotech). Cell preparations either contained Matrigel plus PSC as vehicle controls or $5x10^6$ epicardial cells or $10x10^6$ cardiomyocytes or the combination of $5x10^6$ epicardial cells and $10x10^6$ cardiomyocytes in matrigel/PSC.

Mycoplasma screening was performed on all cells on a regular basis and found to be negative.

Generation and functional assessment of 3D-EHT. In order to cast the tissue constructs, wells were fabricated using polydimethylsiloxane (PDMS) (PDMS, Sylgard 184; Dow Corning, Midland, MI). PDMS linker and base were mixed in a 1:10 mass-ratio and poured in laser-etched acrylic negative templates featuring 4 wells measuring 3x8x2 mm and containing a 1 mm diameter post positioned at 1.5mm from each end. The PDMS was baked at 65°C overnight, removed from the negatives, and then autoclaved. Prior to casting the tissues, the PDMS wells were treated with 5% pluronic acid F127 solution (Sigma, P2443) for 1 hour.

Cardiomyocytes used for construct studies were frozen down on day 21 of the differentiation and given 5 days in culture to recover. During construct casting, cardiomyocytes and epicardial cells were trypsinized and mixed in a collagen gel containing 10x RPMI-1640 medium (Sigma), NaOH, geltrex (Invitrogen, A1413202), collagen I Rat Protein (Gibco Life Technologies, A1048301) and water. The cell-gel solution was poured into the PDMS wells and allowed to solidify for 30 minutes at 37°C. Each construct contained either $5x10^5$ cardiomyocytes alone or $5x10^5$ cardiomyocytes plus $5x10^4$ supportive cells. Constructs were then fed with 7ml of RPMI media plus B27 plus insulin every other day, and spontaneous contractions were observed within 7 days. All constructs were cultured for 14 days, fixed with 4% paraformaldehyde, treated with 30% sucrose at 4°C overnight and finally cryoembedded and sectioned.
Myofibril alignment was quantified using an orientation correlation function (OCF) as previously described[5]. $OCF = 0.5 (\cos (2\theta) + 1)$, where θ is the difference between the angle of the myofibril fibril and the longitudinal cardiomyocyte axis.

For assessment of Ca^{2+}-handling 14 day-old constructs were incubated with fluo-4, AM (Invitrogen, Molecular Probes) for 20 minutes at 37°C. Videos were taken with a Sony Handycam (Vixia HFS20) attached on a fluorescent microscope (Nikon Eclipse TS100). Videos were subsequently converted to frames, imported and analysed using Image J software, normalising the Ca^{2+}-signal to baseline.

Force measurement of constructs was performed after 2 weeks in culture as previously described[6]. In brief, constructs were removed from the PDMS wells and

suspended between a force transducer (Aurora Scientific, model 400A) and length controller (Aurora Scientific, model 312B). To assess the Frank-Starling relationship, constructs were stretched from their resting length to an additional 25% strain in 6 steps while being bathed in a HEPES-buffered Tyrode solution held at 37°C. Force traces were first recorded without electrical stimulation and subsequently with 1, 1.5, 2 and 3 Hz at 5V and 50ms pulse duration. Passive tension and active force traces were recorded and analysed using customized LabView and MATLAB software. A total of 9 independent constructs were generated for each group and used for morphometric and functional analysis. The only exclusion criterion was tissue damage of the integrity of the loop regions. Cardiomyocytes were used from one frozen batch to ensure constant ultra-high purity and epicardial cells were used from three different differentiations.

Gene expression analysis. EHTs were dissociated and gene expression analyses performed after 2 weeks in culture. Total cellular RNA was extracted with ARCTURUS® PicoPure® RNA Isolation Kit (Applied Biosystems, KIT0103) with the following modifications. EHTs were placed in Lysing Matrix D beads (MP Biomedicals, 116913050) and 200µl Extraction Buffer and homogenized using a FastPrep-24™ 5G Instrument (MP Biomedicals). The resulting lysate was transferred to a fresh tube and incubated at 42°C for 30min. 70% ethanol was added to the RNA lysate and loaded into a pre-conditioned column. All subsequent steps were performed according to the supplier's recommendations, including DNase I treatment. 10µl of eluted RNA (corresponding to 100-150ng) were subjected to reverse transcription using Maxima First Strand kit (Thermo, K1641) according to the manufacturer's protocol. Quantitative real-time reverse transcription PCR (RT-qPCR) was performed with Fast SYBR Green Master Mix (Thermo, 4385610) using 5ng of cDNA and 100nM forward and reverse primers. Reactions were run on a 7900HT Fast Real-Time PCR System (Applied Biosystem, 4329001), and data was analyzed using the ΔΔCt method using HPRT1 as the housekeeping gene. Primers were designed using PrimerBlast and confirmed to amplify a single product. A complete list is provided in Supplementary table 3.

Injection of hPSC-derived epicardial cells in chicken embryos. Chicken (*Gallus gallus domesticus*) eggs (Winter Egg Farm, Cambridge, UK) were incubated in a digital cabinet incubator (OVA Easy 380, Brinsea) at 38 degrees. At Hamburger

Hamilton developmental stage 19 (HH19), the eggshell was fenestrated, the window covered with parafilm (VWR) and eggs were placed horizontally in the incubator. At HH35, epicardial cells were transplanted in PSC with matrigel (1:2 dilution) onto the chorionic chicken vasculature. HESCs were fully differentiated to epicardial cells before administration into the chicken embryos. The eggs were then returned in the incubator until stage HH40. The matrigel plugs were harvested and fixed in 4% paraformaldehyde before being stained with anti-HLA1 (abcam) anti-SM22alpha (Abcam) and sambucus nigra lectin (Vector laboratories).

Myocardial infarction and cell transplantation. All studies were approved by the University of Washington Animal Care and Use Committee (IACUC; protocol number 2225-04) and were conducted in accordance with US NIH Policy on Humane Care and Use of Laboratory Animals. The study design comprised two feasibility studies and one definitive study. The first study was designed to assess the acute survival and fate of hESC-EPI. Animals either received $2x10^6$ (*n*=4) or $4x10^6$ (*n*=4) epicardial cells or a vehicle control injection (*n*=4). In a second feasibility study, designed to assess long term survival of epicardial cells and their function animals randomly received either a $6x10^6$ epicardial cells (*n*=6) or a vehicle control injection (*n*=4). The definitive study was conducted to assess the trophic effect of the epicardium on cardiomyocytes. The definitive study design comprised the following four study arms: $5x10^6$ epicardial cells (*n*=15), $10x10^6$ cardiomyocytes (*n*=14), $5x10^6$ epicardial cells plus $10x10^6$ cardiomyocytes (*n*=14) or vehicle control (*n*=13). Animal deaths and cellular engraftment is presented in supplementary table 4.

The protocol for cell implantation has been previously detailed[7, 8]. In brief, male athymic Sprague Daley rats (Harlan/Envigo) underwent anaesthesia through intraperitoneal injection of 68.2 mg/kg Ketamine and 4.4 mg/kg Xylazine, intubated and mechanically ventilated with room air and supplemented oxygen. A second dose of Ketamine and Xylazine was administered 20 minutes later. Animals were placed on a heating pad connected with a rectal temperature probe, which ensured maintenance of body temperature at 37°C. A thoracotomy was subsequently performed, the anterior surface of the heart was exposed and the left anterior descending (LAD) coronary artery was visualized. The LAD was consequently ligated for 60 minutes after which the ligation was removed, the animals reperfused and the chest aseptically closed. Four days post myocardial infarction animals were

anesthetized with Isoflurane before undergoing a second thoracotomy for intramyocardial cell transplantations. Animals were subsequently randomly assigned to one of the treatment groups and cells were injected into the infarct zone. The chest was subsequently closed and the animals were postoperatively monitored.

To optimize graft retention animals received a subcutaneous injection of 5 mg/kg Cyclosporine A on the day before surgery until 7 days after the surgery. To assess cell proliferation in the cell grafts animals were injected with of 50 mg/kg BrdU on days 1 4, 7, and 14 post cell injection. The cohort of animals that was followed up for three months additionally received one BrdU injection 24 hours before the termination of the study.

Echocardiography. All animals underwent echocardiographic exams at baseline before myocardial infarction, 4 days after the infarct and 28 days after cell transplantation. A subset of animals were maintained and imaged at 84 days post-transplantation. Briefly, animals were lightly anesthetized with inhaled isoflurane (Novaplus) and scanned by transthoracic echocardiography (GE Vivid 7) using a 10S (10MHz) paediatric probe. The endpoints acquired comprised fractional shortening (%), left-ventricular diastolic dimension (LVEDD) and left-ventricular systolic dimension (LVESD). LVDD and LVESD are expressed in millimetres (mm). The images were anonymised and a primary reader made measurements in a blinded manner. For validation purposes an independent investigator analysed a sample set of images in a blinded fashion prior to analysis of the entire dataset and at the end to ensure consistency in measurements. The respective Bland-Altman plots and Intra Class Correlation Coefficients of these two tests are presented in Supplementary Figure 20. Details of histologic and echocardiographic parameters are presented in Supplementary table 1.

Immunocytochemistry and Immunohistochemistry. For immunocytochemistry, cells were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS) and blocked in 3% BSA/PBS for 45 minutes at room temperature. Primary antibody incubations were performed at 4°C overnight. The next day, cells were washed and incubated with Alexa-Fluor conjugated secondary antibodies for 45 minutes at room temperature (RT) before staining with 49,6-diamidino-2-phenylindole (DAPI) for 10 minutes to visualize the nuclei. For

immunohistochemistry (IHC), hearts were excised post mortem and prepared as described[8]. Briefly, hearts were washed in PBS, kept in saturated KCl for 20 minutes and subsequently fixed in 4% paraformaldehyde and were paraffin sectioned (5 mm). For IHC stainings, slides were deparafinized, underwent heat-mediated antigen retrieval for 15 minutes and blocked with 5% BSA/PBS containing 0.3% Triton X-100 for one hour at RT, followed by incubation with primary antibodies at 4°C overnight and fluorescent secondary antibodies were applied at room temperature for 60 minutes on the consecutive day. All antibodies used for immunocytochemistry and immunohistochemistry studies are detailed in Supplementary table 5.
For quantification of sarcomeric length, a total number of 1271 sarcomeres (129 cardiomyocytes) were quantified *in vitro* (9 constructs) and 4660 sarcomeres (407 cardiomyocytes) were quantified *in vivo* (37 animals) by manual measurements in a blinded fashion.

Infarct and graft quantification. To assess infarct size, slides were stained with picrosirius red/ fast-green stain. Subsequently in the infarcted sections, picrosirius red positive area was quantified and normalized to the left ventricular area in each section. For quantification of cardiac graft size, slides were stained overnight with human mitochondria antibody (Novus) and α-Actinin (Abcam) to quantify the size of the human cardiac grafts followed by 1 hour incubation with Alexa Fluor-488 donkey anti-rabbit and Alexa Fluor-568 goat anti-mouse secondary antibodies (Invitrogen). The corresponding graft size was then normalized to the size of the infarct area. All animals were used for analysis except one animal in the CM only study arm, which didn't exhibit a detectable graft. Images were acquired on a Nikon TiE Inverted Widefield Fluorescence High-Resolution Microscope. To assess epicardial grafts, anti-GFP (Novus) and anti-human Mitochondria (Novus) antibodies were used. For investigation of epithelial to mesenchymal transition of grafted epicardial cells, slides were stained with antibodies directed against GFP (Novus), Vimentin (Dako) and Wide-spectrum Cytokeratin (Dako). To determine the fate of epicardial cells, slides were co-stained with antibodies directed against human Mitochondria (Novus) and cardiomyocyte (alpha-Actinin (Abcam)), endothelial cells (human Lectin (Ulex europaeus, Vector)), smooth muscle cells, (Smooth Muscle α-Actin (Dako)), or fibroblasts (S100A4 (Abcam)). To detect cardiac grafts, antibodies directed either against human mitochondria and α-Actinin or against β-MHC (Developmental Studies

Hybridoma Bank) were used. For assessment of microvascular density, slides were stained with CD31/PECAM (Novus) and either β-MHC (Developmental Studies Hybridoma Bank) or cTnI (Abcam). For quantification of microvascular density in cardiac grafts, the infarct zone and the non-injured border zone, 9 high power images were taken in each of the three areas of interest. The number of lumen was counted and expressed as vessel number/ area (mm^2). All images were acquired in technical replicates per animal on a Zeiss LSM700 microscope using ZEN software and were subsequently analysed using Image J software. A detailed description of the antibodies and dilutions is provided in supplemental online table 1.

RNA-sequencing. The starting material for RNA sequencing was hESC-derived epicardium as used for all *in vitro* and *in vivo* experiments. For bulk RNA-sequencing total RNA was extracted with the RNeasy Mini kit according to the manufacturer's instructions (Qiagen). This was followed by RNAse treatment to remove contaminating DNA. Samples were consecutively sent to the Genomics facility at the Wellcome Trust - Medical Research Council Cambridge Stem Cell Institute, where cDNA libraries were generated using the SMARTer Stranded Total RNA-Seq Kit.

All samples were sequenced on two lanes of Illumina HiSeq2500. Short reads were mapped to the *Homo Sapiens* genome GRCh38 using HISAT2[9]. For each sample, the bam files corresponding to both lanes were merged with bamtools[10]. This data has been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE122714 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE122714)[11].
The number of reads per sample vary from 10 millions (NC1) to 24 millions (NC2). Quality control and read count was performed with SeqMonk (https://www.bioinformatics.babraham.ac.uk/projects/seqmonk/). Differential Expression analysis between differentiated epicardium and NC cells was performed with DESeq2[12]. The list of 8261 differentially expressed genes was filtered to retain: 1) only genes encoding putatively secreted proteins, according to the Human Protein Atlas (http://www.proteinatlas.org/humanproteome/secretome,[9]). 2) only genes annotated with the gene ontology term “extracellular matrix organisation” using QuckGO (https://www.ebi.ac.uk/QuickGO,[13]). The heatmap only displays differentially expressed genes with an adjusted p-value < 1e-7. It was performed using the function heatplot of the package made4 on the log2 of reads per million reads

values[14]. For clustering, heatplot uses Pearson correlation distances and the average agglomeration method. The GO enrichment was performed with WebGestalt[15]. A complete list of the genes and their expression is shown in supplementary table 2.

Statistics. All *in vitro* studies were performed as three biological replicates (independent experiments performed on different days), each of which was performed using 3 technical replicates. All *in vivo* data specifically state the number of animals assessed for each time point. The normal distribution of our values was confirmed using the D'Agostino & Pearson omnibus normality test where appropriate. Variance between samples was tested with the Brown-Forsythe test. Statistical testing was performed using an unpaired t-test for two group comparisons and a paired t-test for comparison of two paired groups. For multiple-group comparison a one-way ANOVA with a post-hoc Tukey test was used if the group variance was equal and a Kruskall-Wallis test with Dunn's correction for multiple comparisons was applied for groups with unequal variance. Measuring two-sided significance, a p-value of 0.05 was considered statistically significant. All analysis was performed using GraphPad Prism software in a blinded fashion. All results are expressed as mean± SD., unless otherwise stated.
For all *in vivo* experiments group sizes were estimated based on power analyses using previous study variance. While no formal methods of randomization were used, the animals were randomly selected by a technician who was blinded to treatment. Analysis of all histology slides as well as all functional data were analysed in a blinded fashion. Death was the only exclusion criteria for further histologic and functional analysis. Supplementary table 4 details animal mortality and grafting.

Further details can be found in the Life Sciences Reporting Summary.

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