Epicardial cells derived from human embryonic stem cells augment cardiomyocyte-driven heart regeneration

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Abstract:

The epicardium and its derivatives provide trophic and structural support for the developing and adult heart. Here we tested the ability of human embryonic stem cell (hESC)-derived epicardium to augment the structure and function of engineered heart tissue (EHT) *in vitro* and to improve efficacy of hESC-cardiomyocyte grafts in infarcted athymic rat hearts. Epicardial cells markedly enhanced the contractility, myofibril structure and calcium handling of human EHTs, while reducing passive stiffness compared to mesenchymal stromal cells. Transplanted epicardial cells formed persistent fibroblast grafts in infarcted hearts. Co-transplantation of hESC-derived epicardial cells and cardiomyocytes doubled graft cardiomyocyte proliferation rates *in vivo,* resulting in 2.6-fold greater cardiac graft size and simultaneously augmenting graft and host vascularization. Notably, co-transplantation improved systolic function compared with hearts receiving either cardiomyocytes alone, epicardial cells alone or vehicle. The ability of epicardial cells to enhance cardiac graft size and function make them a promising adjuvant therapeutic for cardiac repair.

Introduction:

Despite major advances in the treatment of heart failure due to systolic impairment, therapeutic approaches have fallen short of addressing the cause of the problem; injury of the mammalian heart leads to irreversible loss of contractile myocardial tissue which is incapable of regeneration. At the turn of the millennium heart failure was widely identified as an emerging epidemic[1]. To date 5.6 million patients in the US alone and 23 million worldwide are suffering from heart failure with 50% dying within 5 years after being diagnosed[2, 3]. Current treatment is limited to ameliorating symptoms and slowing the natural progression of the disease but fails to compensate for the loss of contractile myocardium post-injury.

Regenerative medicine may hold the key to effectively treating heart failure by using stem cell-derived cardiovascular cells and tissues to restore full contractile function[4, 5]. Of all stem cell types, human pluripotent stem cells such as embryonic stem cells (hESC) and induced pluripotent stem cells (hiPSC) have the greatest potential for forming cardiovascular tissues, reliably giving rise to cardiomyocytes[6-8], endothelial cells[9, 10], smooth muscle cells[11] and more recently also epicardial and endocardial-like cells[12-15] under chemically defined conditions. Furthermore, hESC-derived cardiomyocytes have been successfully used to remuscularize infarcted rat [8] and guinea pig[16, 17] hearts, resulting in electrical integration and preserving cardiac function. The clinical potential of this technology has also been demonstrated in non-human primate models, where transplantation of human or monkey pluripotent stem cell (PSC)-derived cardiomyocytes resulted in substantial remuscularization of the infarcted heart and restoration of cardiac function[18-20].

While mammalian heart regeneration has made progress, hurdles remain, such as relative immaturity of transplanted cells, suboptimal graft retention, insufficient cellular proliferation and a small graft size. *In vitro*, hPSC-derived cardiomyocytes derived from most protocols at best resemble cardiomyocytes found in a first trimester fetus, which may limit the functional benefits post-transplantation[21]. To date, little attention has been devoted to a supportive cell type that would promote maturity of hESC-derived cardiomyocytes and increase the graft size post-transplantation *in vivo*.

In early mammalian heart development, the epicardium plays a pivotal role as a progenitor cell source and provides trophic support for developing cardiomyocytes. Because the epicardium gives rise to cardiac fibroblasts and coronary smooth muscle cells, it is essential for the formation of a functioning connective tissue and coronary vasculature[22, 23]. Moreover, epicardium-derived cells are essential for cardiac proliferation and compaction[24-26] and have been reported to promote cardiomyocyte maturation[27-29]. Given its trophic role in heart development we hypothesized that epicardial cells could promote cardiomyocyte maturation and contractility in hESC-based 3D-engineered heart tissues (EHTs) *in vitro* and enhance engraftment and maturity leading to potential functional benefits when co-transplanted with hESC-derived cardiomyocytes *in vivo*.

We report here that hESC-derived epicardium promotes the development of 3D-EHTs *in vitro* and cardiac grafts *in vivo* via cardiomyocyte maturation, proliferation and contraction. In the infarcted heart, hESC-derived epicardial cells (hESC-EPI) also increase endogenous neo-vessel development and enhance hESC-CM proliferation and subsequent maturation, thus creating larger grafts of human myocardium that further enhance ventricular function. By recapitulating key developmental steps, the epicardium augmented cardiomyocyte function, making it a promising adjuvant therapy in regenerative medicine.

Results:

HESC-derived epicardial cells promote cardiomyocyte maturation in 3D-EHT

We first generated hESC-derived GFP-transgenic epicardial cells and wild-type (WT) cardiomyocytes as previously described[8, 12], (Fig. 1a-b). Epicardial cells expressed epicardial and epithelial markers, WT1 and pan-cytokeratin, but no mesenchymal markers such as vimentin after their derivation *in vitro*[12, 30]. The functionality of epicardial cells was initially demonstrated through differentiating them to cardiac fibroblasts *in vitro* under chemically defined conditions that included VEGF and FGF. At the end of this differentiation protocol they expressed the fibroblast and mesenchymal markers, S100A4, DDR2 and vimentin, but lost their epithelial character indicating successful epithelial to mesenchymal transition (EMT). During

epicardial to fibroblast differentiation, WT1 was downregulated while the fibroblast marker S100A4 was gradually upregulated. (Supplementary Fig. 1a-e).

Next, we tested the utility of epicardial cells in the context of 3D engineered heart tissues (EHTs). We incorporated epicardial cells along with hESC-CMs into collagen-based 3D-EHTs[31], which developed for 14 days under passive stress before they were subjected to histological and functional assessment (Fig. 1c-d). To investigate the potency of epicardial cells in 3D-EHT we compared them with constructs containing: (1) cardiomyocytes alone; (2) cardiomyocytes and hESC-derived mesenchymal cells; (3) cardiomyocytes and primary human mesenchymal stromal cells (MSCs) from bone marrow. Both epicardial cells as well as primary mesenchymal cells had the strongest effects on tissue remodelling and compaction whereas tissues containing high-purity cardiomyocytes alone demonstrated minimal compaction (Fig. 1e-g).

Whilst in the tissues, hESC-EPI underwent EMT as indicated by the increase in expression of vimentin and decrease in pan-cytokeratin comparing constructs after 7 days and 14 days of their development. In this context, epicardial derivates have previously been termed epicardium-derived cells (EPDCs)[23]. EPDCs are positive for Vimentin and S100A4, indicative of a fibroblast phenotype (Supplementary Fig. 2a-c).

To determine the state of cardiomyocyte maturity, histological sections of the 3D-EHTs were stained for sarcomeric proteins, and the sarcomere length, cell diameter, cell sectional area and myofibril alignment were quantified. HESC-EPI promoted the greatest sarcomere length, cell diameter, cell sectional area and myofibril alignment, which correlate with cardiomyocyte maturation, compared to primary MSCs, hESC-derived MSCs or CM alone (Fig. 1h-j, supplementary Fig. 3a). 3D-EHTs containing hESC-EPI and primary MSC exhibited the greatest degree of sarcomeric organisation (Supplementary Fig. 3b). Furthermore, constructs containing hESC-EPI expressed more connexin 43, a marker of electrical connectivity between cardiomyocytes, compared to the other groups (Supplementary Fig. 2d-e). Gene expression by qRT-PCR demonstrated trends consistent with increases in the ratios of adult/fetal isoforms of contractile proteins (*MYH7/MYH6*, *MYL2/MYL7* and *TNNI3/TNNI1)*, all indicators of cardiomyocyte maturity (Supplementary Fig. 4). In conclusion, hESC-derived epicardial cells replicate key steps of early embryonic heart development in 3D-EHTs resulting in increased cardiomyocyte maturation.

HESC-derived epicardial cells enhance functional maturation of 3D-EHT

We next tested whether the beneficial effects of hESC-derived epicardial cells observed histologically also translated to an increase in contractility. EHT constructs were transferred to a myograph with a length controller and a force transducer (Supplementary Fig. 5a); constructs containing CM and either hESC-MSC or primary MSC or hESC-EPI exhibited a Frank-Starling relationship, where twitch force increased linearly with increasing preload. In line with the histological finding that high-purity CM alone do not result in efficient tissue remodelling, cardiomyocyte maturation or electrical connectivity, we did not observe coordinated contraction or measurable force production in 3D-EHTs containing CM alone. Constructs containing hESC-EPI produced the greatest total force amongst the 4 groups. Additionally, EHTs containing hESC-EPI showed the greatest increase in active force production with increasing strain (i.e. the greatest contractility) compared to primary MSCs and hESC-MSCs (Fig. 2a, Supplementary Fig. 5b). At the same time, 3D-EHTs containing hESC-EPI produced the least passive force compared to primary MSCs or hESC-MSCs, which would correspond to a more compliant tissue with better relaxation potential during diastole (Fig. 2b).

When assessing the Ca^{2+}-handling of the constructs, those containing hESC-EPI and primary MSCs accounted for the most mature Ca^{2+} transients (Fig. 2c-d). Constructs containing EPDCs had more rapid Ca^{2+}-release and Ca^{2+}-decay compared to those with primary MSCs (Fig. 2e, Supplementary Fig. 5c-d). Constructs containing hESC-MSC displayed irregular and broad Ca^{2+} transients, reflecting less coordinated beating. In those containing CM-only, no coordinated Ca^{2+} transients were detectable, but rather asynchronous contraction of single non-connected cardiomyocytes was observed, which translated to noise in multicellular regions of interest.

Next, we tested if the ability of epicardial cells to promote 3D-EHT function is independent of the parental hESC line. We generated EHTs where cardiomyocytes and EPDCs were differentiated from the same H9 line, as well as with cardiomyocytes from H9 cells and EPDCs from RUES2 hESCs. In both contexts, the EPDC-containing EHTs showed superior contractility and Ca^{2+} dynamics compared to EHTs made with hESC-MSCs or primary MSCs. These experiments demonstrated that enhancement of EHT function by EPDCs is a general attribute of epicardial cells

and not a line-dependent artefact (Supplementary Fig. 6a-b, Supplementary Fig. 7a-d).

Furthermore, in a separate experiment we determined the nature of the non-cardiomyocyte population obtained upon differentiation of high-purity cardiomyocytes by flow cytometry using markers previously described[32]. We found positivity for THY1 and PDGFR-β and to a minor degree for CD31 and NKX2.5 in this cTnT-negative cell population (Supplementary Fig. 8). Overall, this demonstrated a mesenchymal and fibroblast-like nature to these non-cardiomyocytes and was comparable between RUES2- and H9-derived cells.

In conclusion, hESC-derived epicardial cells promote functional maturation of 3D-EHT. These findings encouraged us to explore their use *in vivo* as an adjunct to cardiomyocyte transplantation for cardiac repair.

Epicardial cells engraft and differentiate in the myocardial infarct

To assess the response of hESC-derived epicardial cells to engraftment *in vivo* we performed a series of pilot transplants into the infarct zone of athymic rats (Supplementary Fig. 9a). Because most non-myocytes that are transplanted into the heart rapidly die[33], we subjected the epicardial cells to heat shock and a prosurvival cocktail (PSC) of anti-apoptotic and anti-necrotic factors. At 7 days post-transplantation we found small grafts in 3 out of 4 animals receiving $2x10^6$ cells and larger grafts in all 4 animals receiving $4x10^6$ cells (Supplementary Fig. 9b-c). To maximise survival at 28 days post-transplantation, we delivered $6x10^6$ cells and found large grafts in 6 out of 6 animals (Supplementary Fig. 9d), indicating the grafts survive long term. We confirmed in a separate experiment that delivery with heat shock + PSC is required for engraftment of epicardial cells (Supplementary Fig. 10a-c). Conversely, epicardial cell transplantation in NOD scid gamma mice, without heat shock + PSC, demonstrated no detectable graft formation at 28 days (Supplementary Fig. 11a-c).

At 7 days post-transplantation the EPDCs co-expressed pan-cytokeratin and vimentin, indicating ongoing EMT. At 28 days post transplantation EMT was essentially complete, with all grafted cells expressing vimentin and almost no detectable expression of pan-cytokeratin (Supplementary Fig. 9e-f). A small subpopulation of grafted vimentin-positive cells co-expressed WT1 on day 7 and day

28. This reflects the human fetal heart, where activated vimentin+ epicardial cells invade the compact myocardium with a subpopulation retaining WT1 expression (Supplementary Fig. 9g-g'). At 28 days the grafted epicardial cells expressed S100A4, suggesting a fibroblast phenotype, whereas they neither expressed SM22α nor integrated into blood vessel walls, (Supplementary Fig. 12a-b). Grafted cells were negative for the cardiac marker α-actinin and the endothelial marker human Lectin (Supplementary Fig. 12c-e). Taken together, these data indicate that EPDCs differentiate into cardiac fibroblast-like cells with no formation of cardiomyocytes, endothelial cells or smooth muscle cells.

Because EPDCs readily form smooth muscle cells *in vitro,* we hypothesized that the infarct environment inhibited this differentiation pathway. To test this, we delivered hESC-EPI in PSC onto the chorionic vasculature of chick embryos. After 5 days the human EPDCs had integrated into the walls of host vessels and expressed SM22α (Supplementary Fig. 13a-c). Thus, the epicardial cells are multipotent and in an embryonic environment readily differentiate into vascular smooth muscle, whereas in the adult infarct they form fibroblast-like cells.

Co-transplantation of epicardial cells and cardiomyocytes augments microvascular density

The long-term persistence of hESC-derived epicardial cells in infarcted hearts led us to hypothesize that these cells would exhibit a trophic effect on grafted hESC-cardiomyocytes and the host myocardium. To test this, we performed a co-transplantation study where four groups of athymic rats either received an injection of $5x10^6$ hESC-derived epicardial cells (EPI), $10x10^6$ hESC-derived cardiomyocytes (CM), the combination of both (EPI+CM: $5x10^6$ hESC-derived epicardial cells plus $10x10^6$ cardiomyocytes) or vehicle control (PSC; Fig. 3a). Four weeks post transplantation no difference in infarct size was found between the groups, ruling out effects on infarct scar healing (Fig. 3b-c).

To assess whether cell transplantation had an effect on host vessel recruitment, we quantified the microvascular density in the cardiac grafts, the infarct zone and the non-injured border zone (Fig. 3d). Microvascular density was significantly increased in cardiac grafts of animals that were co-transplanted with epicardial cells and cardiomyocytes. Furthermore, erythrocytes were readily detectable in the lumens of

the vessels, indicating perfusion via the coronary circulation (Fig. 3e, Supplementary Fig. 14a). We also observed an increase in microvascular recruitment in the infarct zone and in the non-injured border zone of the infarct, which was highest in EPI+CM, followed by CM, then EPI and finally vehicle control (Fig. 3f-g). To assess the timing of microvascular sprouting we made use of our pilot trial dataset and demonstrated that, epicardial cells alone lead to a significant increase in microvascular density in the infarct- and borderzone by day 28 but not by day 7 post transplantation, indicating delayed angiogenic effects (Supplementary Fig. 14b-e).

To address the maturity of neo-vessels we also screened the three areas of interest for presence of smooth muscle cell coated arteries. Vessels containing SM22α+ mural cells were abundant in the infarct zone and the border zone of all groups. However, when assessing their presence within cardiac grafts we observed smooth muscle cell coated vessels in the EPI+CM group but not in the CM-alone group, suggesting epicardial cells promote smooth muscle encoatment and vascular maturation within the graft (Supplementary Fig. 14f-k). Absence of co-staining for SM22α and human mitochondria further confirmed that the smooth muscle coats were rat-derived, and that epicardial cells were also not able to differentiate to smooth muscle cells in the infarct zone in the presence of cardiomyocytes (Supplementary Fig. 14l). In summary, hESC-derived epicardial cells create a more highly vascularized cardiac graft, and microvascular density surrounding scar and border zone, which should promote a more favourable niche for hESC-cardiomyocyte engraftment and function.

Co-transplantation promotes cardiac graft size, proliferation and maturity

We next assessed the effects of epicardial cells on the cardiac grafts. Given the trophic effects of epicardial cells on cardiomyocytes *in vitro* we first investigated whether co-transplantation would affect cardiac graft size. Cardiac grafts were readily identified with antibodies directed against β-MHC (MYH7; whereas rat cardiomyocytes predominantly express α-MHC/MHY6) and co-expressed α-actinin. Cardiac grafts were 2.6-fold larger when cardiomyocytes were co-transplanted with epicardial cells, compared to cardiomyocytes alone, averaging 3.9±1.6% of the left ventricle vs 1.5±0.9% of the left ventricle, respectively (Fig. 4a-b; Supplementary Fig. 14m).

Because epicardial cells secrete growth factors for cardiomyocytes during development, we hypothesized that epicardial cells augmented graft size via increased cardiomyocyte proliferation. All rats in this phase of the study were pulsed with the thymidine analogue, BrdU, on 1, 3, 7 and 14 days post-cell delivery. To determine cumulative proliferation rates in the grafts we stained with antibodies directed against BrdU and the human-specific cardiac marker β-MHC. The proliferative index of β-MHC positive cells was 2-fold higher in animals that received the combination of hESC-EPI and cardiomyocytes (8%±1.4%) compared with cardiomyocytes alone (4±0.9%, $p<0.0001$; Fig. 4c-d). Conversely, there was no difference in the proliferative index of vimentin-positive EPDCs in animals receiving epicardial cells alone or epicardial cells with cardiomyocytes (Supplementary Fig.15a-b). Taking the graft cardiomyocyte BrdU rates as daily averages of DNA synthesis, and assuming that the cell cycle lasts 24 hours, hESC-CM graft expansion can be calculated for each group. For 28-day grafts, control expansion would be 1.04^28, or 3.0-fold expansion, while in co-transplantation studies it would be 1.08^28, or 8.6-fold expansion. This predicts a 2.9-fold difference in graft size, which is quite close to our observed 2.6-fold difference. Thus, although we cannot rule out a role for increased graft survival, these data indicate that enhanced cardiomyocyte proliferation is a major driver for the increased cardiomyocyte graft size observed with hESC-derived epicardial cell co-transplantation.

Given the epicardial effects on cardiomyocyte maturation *in vitro,* we assessed the sarcomere length of the cardiac grafts. In line with our findings *in vitro,* cardiomyocytes that were co-transplanted with epicardial cells exhibited a greater sarcomeric length and cell diameter as well as a 77% larger cell sectional area (Fig. 4f-h) than those that were transplanted alone, indicative of a more mature phenotype. Additionally, we demonstrated that co-transplantation of cardiomyocytes with epicardial cells leads to an isoform switch from ssTnI to cTnI in cardiac grafts (Fig. 4e).

As cardiac fibrosis impedes structural integration of grafts and host[34], we were concerned that epicardial cell-derived fibroblasts might interfere with gap junctions between graft and host cardiomyocytes. To investigate this, we performed combined immunostaining for β-MHC, cTnT and the gap junction protein connexin43. Gap junctions between graft and host were seen in multiple areas across all animals,

demonstrated by connexin43 expression between neighbouring human and rat cardiomyocytes (Fig. 4i). While, epicardial cells themselves can express connexin43[35, 36], the gap junctions identified in our study were predominantly between cardiomyocytes. In conclusion, hESC-derived epicardial cells promote cardiac graft size, in part through proliferation, and they enhance maturation/myofibril development, while still permitting structural integration of grafted cardiomyocytes with the recipient myocardium.

Co-transplantation promotes cardiac function

To assess the functional effects of cardiac grafts on global host heart function, we performed cardiac ultrasound on all animals prior to infarction, immediately before cell injection and after 28 days of follow-up. The ultrasound scans (AM) and their interpretations (JB and FW) were performed by investigators who were blinded to the treatment to prevent bias. All groups exhibited comparable left ventricular (LV) dilation and decline in LV function after infarction, consistent with the histological finding of comparable infarct sizes (Fig. 5a-c; supplementary table 1). Compared with their pre-injection (post-infarction) baseline values, the vehicle control group displayed a 9±5.4% decline in fractional shortening by 4 weeks after injection, indicating a progression towards heart failure ($p<0.0001$ vs. baseline). The EPI group showed a 5.3±6.5% decline in fractional shortening. Similar to previous reports from our group[8], transplantation of cardiomyocytes-only preserved cardiac function over the 4-week period, indicating that CM transplantation prevented the progression to heart failure. Importantly, animals receiving combined CM+EPI grafts showed a 4.5±3.6% improvement in fractional shortening, indicating that co-transplantation significantly improved LV function ($p=0.0175$ vs. cardiomyocytes-only). Furthermore, we demonstrated that in the CM and CM+EPI groups, improvements in LV function correlate linearly with graft size (Supplementary Fig. 15c).

Comparing pre- and post-injection values, left-ventricular end-systolic dimension (LVESD) remained stable in the CM+EPI group and increased in the CM only group, followed by greater increases in the EPI and the vehicle control group. The change in LVESD in the CM+EPI group was significantly smaller than in the vehicle control group but the difference to the CM only group or the EPI group did not reach statistical significance (Fig. 5d-e). For left ventricular end diastolic dimension (LVEDD), ANOVA did not yield a significant difference (Fig. 5f-g). Taken together,

these studies show that co-transplantation of hESC-derived epicardial cells with cardiomyocytes leads to a greater increase in cardiac function compared to transplantation of cardiomyocytes alone. Furthermore, this benefit manifests principally as enhanced systolic function rather than impacting left ventricular remodelling.

To investigate whether these effects would be present in the long term, we followed a subset of 4-5 animals per group for up for 3 months. Three months after cell grafting hESC-EPI as well as hESC-derived cardiomyocytes were still present in the infarct zone as confirmed by anti-human mitochondrial staining (Supplementary Fig. 16a-c). In line with the 28-day follow-up, at 84 days post transplantation no differences were found in infarct size between the four study groups (Supplementary Fig. 16d). Functional analysis demonstrated long-term perpetuation of benefits perceived at 1 month due to persistent systolic improvement. Fractional shortening showed no decline in animals receiving CM+EPI or CM-only but a significant decline in animals receiving either EPI or vehicle control. While there was no difference in the change of fractional shortening between day 28 and day 84 among the groups, the change occurring between day 4 and day 84 was significantly greater in the CM+EPI group compared to CM only or EPI only or vehicle control (Supplementary Fig. 16e-f). Indeed the 4.5% improvement in FS of epicardial cell co-therapy over cardiomyocytes alone seen at 4 weeks increased further to 9.6% by 12 weeks (Supplementary Fig. 16f). To summarise, hESC-derived cellular grafts and related functional improvement in cardiac function persist in the long term.

RNA-sequencing reveals the epicardial secretome

Finally, to address the question of putative mediators of epicardial cell-driven cardiac repair we performed RNA sequencing of hESC-derived epicardium as used for all *in vitro* and *in vivo* experiments. In embryonic heart development, the neural crest (NC) is essential for pharyngeal arch organisation and outflow tract septation but lineage tracing studies have to date not suggested a role in cardiac maturation[37-39]. We first used hESC-derived NC cells in 3D-EHTs, demonstrating the inability of this cell population to result in structural and functional heart maturation, in contrast to epicardial cells (Supplementary Fig. 17a-b). We therefore used NC cells as a negative control for RNA-sequencing (Fig. 6a, Supplementary table 2).

As epicardial cells resulted in stark effects on tissue remodelling of 3D-EHTs and because it has been demonstrated that the extracellular matrix plays a key role in epicardial-driven heart repair, we focused on extracellular matrix molecules secreted by epicardial cells and differentially expressed in the hESC-derived NC cells (Supplementary table 2). The heatmap shown in Figure 6a displays differentially expressed genes with an adjusted p-value $<1x10^{-7}$ (a complete list of the genes and their expression is shown in supplementary table 2). Amongst them, Fibronectin is one of the most differentially expressed candidates. We then performed a gene ontology (GO) enrichment analysis, which further highlights a potential role for ECM expressed by hESC-EPI in myocardial development and maturation (Fig. 6b-c, Supplementary Fig. 18).

We first demonstrated that Fibronectin was also abundantly expressed at the protein level in 3D-EHT containing epicardial cells but to a lesser degree in constructs containing hESC-MSC, primary MSC or cardiomyocytes alone (Fig. 6d). Furthermore, Fibronectin was highly expressed in cardiac grafts *in vivo* in animals receiving epicardial cells and cardiomyocytes or epicardial cells alone but only at a rudimentary level in those receiving cardiomyocytes alone or vehicle control (Fig. 6e). In summary, RNA-sequencing has provided the secretome of hESC-derived epicardium and identified a potential involvement of extracellular matrix remodelling in the process of cardiac maturation in vitro and in vivo. These data provide a valuable target library for future cardiac repair strategies.

Discussion:

This study was designed to address two gaps in knowledge: the immaturity of hPSC-derived cardiomyocytes, and the inefficient remuscularization of infarcts following hPSC-CM transplantation. Because of the key trophic role played by the epicardium during development, we hypothesized that hPSC-epicardial cells would promote cardiomyocyte proliferation and maturation, resulting in better EHT formation *in vitro* and better infarct remuscularization *in vivo.* We found that hESC-derived epicardial cells undergo EMT to fibroblast-like cells both *in vitro* and *in vivo*. In EHTs EPDCs augment tissue structure and function, resulting in increased cardiomyocyte size, sarcomere length, force production and augmented Ca^{2+} handling. When co-delivered with hESC-CMs into myocardial infarcts, EPDCs stimulate hESC-CM proliferation, increasing remuscularization by 2.6-fold. Additionally, EPDCs stimulate

vascularization within the graft, infarct scar and in the borderzone host myocardium. These changes are accompanied by remodelling of the ECM, including high levels of fibronectin deposition. Finally, co-delivery of hESC-derived epicardium with hESC-CM results in significantly improved ventricular function post-infarction.

The pivotal role of the epicardium in heart development is well recognized. During early embryonic heart formation the epicardial tissue gives rise to coronary smooth muscle cells[22, 23] and myocardial fibroblasts[24, 25, 40]. While smooth muscle cells are critical for formation of the coronary vasculature, cardiac fibroblasts are essential for myocardial proliferation and compaction[26]. Conversely, inhibiting proepicardial outgrowth results in pathologic formation of the coronary vessels and non-compaction cardiomyopathy[41]. The trophic effect of quail and rat epicardium-derived cells has been demonstrated in co-culture experiments resulting in structural and functional maturation of cardiomyocytes[27, 29]. Our data show that co-culture of hESC-derived epicardial cells and cardiomyocytes results in compaction and structural as well as functional maturation of 3D-EHT. More specifically we demonstrate that hESC-derived epicardium outcompetes both hESC-derived MSCs as well as primary MSCs in terms of force generation and Ca^{2+}-handling, corroborating the functional role of its embryonic identity. The functional potency of epicardial cells might prove broadly applicable to current tissue engineering strategies, that would benefit from enhanced structural integrity and function of cardiomyocytes[42].

In neonatal mice and in zebrafish, the epicardium is thought to have a key role in facilitating myocardial regeneration following injury[43, 44]; in contrast the adult mammalian heart displays inadequate epicardial activation and fails to regenerate myocardium post-injury. In this context it has been demonstrated that embryonic cardiac fibroblasts induce greater cardiac proliferation than their adult counterparts[26]. Consequently, we propose that the fetal-stage epicardium generated by hESCs may preferentially promote regeneration.

It is important to consider the mechanism of action through which the epicardial cells improve the impact of cardiomyocyte transplantation. Since cardiomyocytes are already effective by themselves, the 2.6-fold enhancement of cardiomyocyte engraftment induced by epicardial cell co-delivery may underlie much of the benefit on cardiac function. The positive correlation of cardiac graft size and delta FS (%)

corroborates this interpretation (Supplementary Fig.15c). Most of this increase in engraftment can be accounted for by the 2-fold increase in cardiomyocyte DNA synthesis rates induced by epicardial cell co-delivery, although beneficial effects on cardiomyocyte survival cannot be ruled out. Another important factor, however, is that the EPDCs induced a significant amount of angiogenesis in the graft, infarct scar and boderzone host myocardium. Increased vascularization should improve the function of both graft and host tissues and could contribute to the beneficial effect.

A third potential mechanism was raised by our RNA-seq studies, which demonstrated that hESC-derived epicardial cells synthesize an embryonic ECM that is particularly rich in fibronectin. It has been demonstrated that the secretion of fibronectin by epicardial cells is required for heart regeneration in zebrafish[45]. In line with these findings it was demonstrated that the orchestrated secretion of fibronectin, collagen and heparin-binding EGF-like growth factor by embryonic but not adult fibroblasts resulted in cardiomyocyte proliferation[26]. We therefore propose that the matrix laid down by hESC-derived epicardial cells is likely to exhibit developmental cues that are absent in mature post-infarct myocardium, providing an advantageous niche in a hostile environment and the GO analyses of RNA sequencing data are in support of this. Further elucidation of the observed crosstalk between epicardium and cardiomyocytes might aid ongoing tissue engineering and cell therapy endeavours and the extensive data on epicardially-expressed genes made available here provides a rich resource for further study. These mechanisms are not mutually exclusive and could co-exist.

Finally, although we favour the notion that the salutary effects of co-delivering cardiomyocytes plus epicardial cells results from the impact on cardiomyocyte graft size, vascularization and ECM, we cannot rule out a paracrine effect from simply increasing the number of delivered cells in the combined cell group. Indeed, our data do not permit distinguishing the direct mechanical effects of the cardiomyocyte grafts from the paracrine factors they secrete; presumably both mechanisms may co-exist and be augmented by larger cardiomyocyte grafts. However, this does not detract from our primary conclusion that hESC-derived epicardial cells recapitulate their embryonic role in the post-MI setting by promoting hESC-derived cardiomyocyte proliferation and maturity and at the same time favourably influencing host tissue regeneration.

Two other pioneering studies have demonstrated that human primary epicardial cells improve function of the infarcted heart and that co-transplantation of adult cardiovascular progenitors with epicardial cells exerts a synergistic effect that exceeds that of monotherapy[33, 46]. There were several important differences with our study, including their use of epicardial cells and cardiac progenitor cells derived from the adult human heart (vs. our use of hESC derivatives) and their use of NOD-SCID mice with permanent coronary ligations (vs. our use of athymic rats with ischemia-reperfusion injury). While these results demonstrated a beneficial effect of poly-cell therapy, the authors did not detect stable grafts in any of the groups, indicating that the effects seen were paracrine in nature. Indeed, we are not aware of any studies in the literature that have demonstrated robust cardiomyocyte and supportive cell type engraftment in the long term following myocardial infarction. In contrast, our grafts, both cardiac as well as epicardial, and related functional benefits were detectable up to three months post transplantation, providing evidence for longevity and potential perpetuation of benefits in the long term.

In conclusion, hESC-derived epicardial cells are a promising tool to advance regenerative cardiovascular medicine, including cell transplantation as well as tissue engineering strategies. Future studies are warranted to better understand the mechanisms through which epicardial cells propagate the observed benefits and investigate their function in models that more closely match clinical application.

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Author contributions: JB: Principal experimentalist, study design and conceptualisation, data acquisition and interpretation, production of figures, manuscript writing; LPO: tissue culture, 3D-EHT generation, force measurement, assistance during surgery; MC: tissue culture, 3D-EHT generation; HD: force measurement and QRT-PCR; PH: preparation of cell suspension on the day of transplantation, necropsy, assistance during surgery, postoperative animal care; SB: casting of 3D-EHT, force measurement; LG: tissue histology, immunofluorescence, sample preparation for RNAseq; NLN: bioinformatics analysis; DI: conceptual ideas, critical revision of the manuscript for important intellectual content; FS: Critical revision of the manuscript for important intellectual content; FW: functional analysis of echocardiographs; AB: gene expression analysis; AL: experimental guidance and force measurement data analysis; WGB: data interpretation and logistics; AM: animal surgery and logistics; NF: processing of histologic tissue, preparation of slides; MR: force measurement equipment; MRB: critical revision of the manuscript for important intellectual content; CEM: design and concept of the study, obtaining research funding, study supervision, editing and final approval of the manuscript; SS: design and concept of the study, obtaining research funding, study supervision, interpretation of data, editing and final approval of the manuscript.

Competing financial interest: A patent has been filed on the cardiac application of epicardial cells, on which CEM, SS and JB are co-inventors (WO2018170280A1). CEM is a scientific founder and equity holder in Cytocardia.

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Figure Legends:

Figure 1. Generation and maturation of 3D-EHT using hESC-derived epicardial cells and cardiomyocytes.

(a) Epicardial cells derived from hESCs expressing the epicardial markers BNC1 and WT1. Scale bar: 50µm. **(b)** Purity of epicardial cells and cardiomyocytes by flow cytometry. Control groups represent secondary and isotype antibodies for epicardial cells and cardiomyocytes respectively. Flow cytometric analysis was independently repeated 3 times with similar results. **(c)** Schematic of experimental design. Epicardial cells and cardiomyocytes were derived from hESCs and co-cultured in 3D-EHT. **(d)** Schematic of 3D-EHT using hESC-derived epicardial cells and cardiomyocytes. **(e-f)** Compaction and ultrastructure of 3D-EHT containing CM alone, CM+hESC-MSC, CM+Primary MSC or CM+hESC-EPI. Scale bars: 2.5mm and 25µm. (a, e-f) Experiments were independently repeated 9 times with similar results. **(g-j)** Quantification of tissue remodelling, sarcomeric length, cell diameter and cell sectional area.

Mean values; error bars represent SD. Two-sided *p*-values were calculated using a one-way ANOVA with post-hoc correction for multiple comparisons. CM alone,

CM+hESC-MSC, CM+Primary MSC and CM+hESC-EPI, *n*=9, 9, 8, 9 constructs, generated independently and measured on three different days.

Figure 2. HESC-derived epicardial cells promote contractility and Ca^{2+}-handling of 3D-EHT. (a) Active force generation of 3D-EHT containing CM alone, CM+hESC-MSC, CM+Primary MSC or CM+hESC-EPI. Displayed are the Frank-Starling curve of active force production and the slope of the generated curve, respectively. **(b)** Passive force of 3D-EHT containing CM alone, CM+hESC-MSC, CM+Primary MSC or CM+hESC-EPI. Displayed are the curve of passive force production and the slope of the generated curve respectively. **(c)** Representative Ca^{2+} traces of 3D-EHT. **(d)** Overlay of representative Ca^{2+} curves. **(e)** Slope of Ca^{2+}-upstroke and Ca^{2+}-downstroke. Mean values; error bars represent SD. Dotted lines represent 95% confidence intervals. Two-sided *p*-values were calculated using a one-way ANOVA with post-hoc correction for multiple comparisons. CM alone, CM+hESC-MSC, CM+Primary MSC and CM+hESC-EPI, *n*=9, 9, 8, 9 constructs, generated independently and measured on three different days.

Figure 3. Co-transplantation of hESC-derived epicardial cells with cardiomyocytes promotes microvascular density. (a) Schematic of study design. **(b)** Representative Picrosirius Red-Fast Green counterstained infarcted rat heart sections. **(c)** Quantification of myocardial infarct size. **(d)** Schematic of areas assessed for vascularisation. **(e)** Microvascular density in cardiac grafts. **(f)** Microvascular density in the infarct zone. **(g)** Microvascular density in the non-injured border zone of the infarct. Due to the presence of erythrocyte autofluorescence all quantification was performed manually to avoid erroneous detection with automated software.
Mean values; error bars represent SD. Two-sided *p*-values were calculated using a one-way ANOVA with post-hoc correction for multiple comparisons unless otherwise stated. *N*=36 in total for histologic analysis at the 1-month time point. Control, EPI, CM, CM+EPI, *n*=9, 10, 8 and 9 animals. Scale bars: 50µm.

Figure 4. HESC-derived epicardial cells potentiate cardiac regeneration. (a) Representative sections of infarcted hearts demonstrating the cardiac grafts in animals that received either hESC-derived epicardial cells and cardiomyocytes or

cardiomyocytes alone. βMHC is specific for grafted human cardiomyocytes while cTnT antibody stains both rat and human cardiomyocytes. Scale bar: 2.5mm. **(b)** Quantification of cardiac graft size. **(c)** Proliferative index of human cardiomyocytes in cardiac grafts. Scale bar: 20μm. **(d)** Quantification of proliferative index. **(e)** Isoform switch of SSTnI to cTnI in cardiomyocytes *in vivo* in animals receiving EPI+CM and CM alone. Staining performed in 5 animals per group. Scale bars: 20μm. **(f)** Quantification of sarcomeric length. **(g)** Quantification of cell diameter in cardiomyocytes *in vivo*. **(h)** Quantification of cell sectional area in cardiomyocytes *in vivo*. **(i)** Cardiac grafts and Cx43+ gap junctions with host tissue. Scale bars 50μm for (i) and 20μm for (*) and (**). (i) CX43 staining was performed on all animals. Mean values; error bars represent SD. Two-sided *p*-values were calculated using an unpaired t-test unless otherwise stated. *N*=37 in total for histologic analysis after 1 month; Control, EPI, CM, CM+EPI, *n*=9, 10, 9 and 9 animals.

Figure 5. Co-transplantation of epicardial cells and cardiomyocytes promotes functional recovery.

(a) Schematic of study design and timing of echocardiographic data collection. **(b)** Echocardiographic effects of hESC-derived epicardial cell augmented cardiac grafts on postinfarct ventricular function. Fractional shortening values are given for the 96hr pre-treatment baseline and 1-month follow-up. **(c)** Difference in fractional shortening. **(d)** Left-ventricular end-systolic dimension (LVESD) are given for the 96hr pre-treatment baseline and 1-month follow-up. **(e)** Difference in LVEDS. **(f)** Left-ventricular end-diastolic dimension (LVEDD) are given for the 96hr pre-treatment baseline and 1-month follow-up. **(g)** Difference in LVEDD.

Mean values; error bars represent SD. Two-sided *p*-values were calculated using a paired t-test for comparison of cardiac function within groups between baseline and 1-month follow-up. If more than 2 groups were compared, a one-way ANOVA with post-hoc correction for multiple comparisons was used. *N*=56 in total for functional analysis after 1 month; Control, EPI, CM, CM+EPI, *n*=13, 15, 14 and 14 animals.

Figure 6. Epicardial secretome. (a) Heatmap depicting differentially expressed secreted factors in epicardial cells compared to neural crest. **(b)** Gene enrichment analysis showing the top enriched GO biological processes. The 705 genes specifically enriched in the EPI secretome were subjected to a hypergeometric test

against a background of the 2413 secretome genes expressed in NC or EPI. GO terms are shaded between black and white based on their false discovery rate after *Benjamini–Hochberg correction*. **(c)** Circular GO plot depicting the top GO terms enriched in EPI vs. NC associated with at least 10 genes on the right and the associated genes on the left. Genes are shaded between black and white based on their fold enrichment in EPI compared with NC. (a-c) EPI and NC, *n*=3 and 3. **(d)** Fibronectin expression in 3D-EHT *in vitro*. CM alone, CM+hESC-MSC, CM+Primary MSC and CM+hESC-EPI, *n*=9, 9, 8, 9 constructs, generated independently and measured on different days. Scale bars 50µm. **(e)** Fibronectin expression in epicardial and cardiac grafts in animals *in vivo*. Scale bars 50µm. This staining was performed on all animals at the 1-month time point (*n*=37).

Online methods:

Online methods have been provided as a separate document with separate references to keep the number of references of the main article below 50.

Data availability:

The raw data that support the findings of this study are available from the corresponding author upon reasonable request.

Methods only references:

Methods only references are provided as a single list at the end of the methods section.