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Endothelial extracellular vesicles contain protective proteins and rescue ischemia-reperfusion injury in a human heart-on-chip

Authors: Moran Yadid^{1,2}, Johan U. Lind^{1,2,3}, Herdeline Ann M. Ardoña¹, Sean P. Sheehy^{1,2}, Lauren E. Dickinson^{1,2}, Feyisayo Eweje^{1,2}, Maartje M.C. Bastings^{2,4,5,6}, Benjamin Pope^{1,2}, Blakely B. O'Connor¹, Juerg R. Straubhaar⁷, Bogdan Budnik⁷, André G. Kléber⁸, Kevin Kit Parker^{1,2*}

Affiliations:

¹Disease Biophysics Group, John A. Paulson School of Engineering and Applied Sciences, Harvard University, Cambridge, MA 02138, USA.

²Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, MA 02115, USA.

³ Department of Health Technology, Technical University of Denmark, Kgs. Lyngby, 2800, Denmark.

⁴Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA.

⁵Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, MA 02115, USA.

⁶Ecole Polytechnique Federale Lausanne (EPFL), Institute of Materials, Programmable Biomaterials Laboratory
Station 12, 1015-CH, Switzerland

⁷FAS Division of Science, Harvard University, Cambridge, MA 02138, USA.

⁸Beth Israel & Deaconess Medical Center, Harvard Medical School, Boston, MA 02115, USA.

*Corresponding author. E-mail: kkparker@seas.harvard.edu

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One Sentence Summary: Endothelial extracellular vesicles containing cardioprotective proteins rescue engineered human cardiac tissue in an ischemia-reperfusion injury model.

Abstract: Extracellular vesicles (EVs) derived from various stem cell sources induce cardioprotective effects during ischemia-reperfusion injury (IRI). These have been attributed mainly to the anti-apoptotic, pro-angiogenic, miRNA cargo within the stem cell-derived EVs. However, EV-mediated endothelial signaling to cardiomyocytes, as well as their therapeutic potential toward ischemic myocardial injury, are not clear. EV content beyond miRNA that may contribute to cardioprotection have not been fully illuminated. This study characterized the protein cargo of human vascular endothelial EVs (EEVs) to identify lead cardioactive proteins and assessed the effect of EEVs on human laminar cardiac tissues (hlCTs) exposed to IRI. We mapped the protein content of human vascular EEVs and identified proteins that were previously associated with cellular metabolism, redox state, and calcium handling, among other processes. Analysis of the protein landscape of human cardiomyocytes revealed corresponding modifications induced by EEVs treatment. To assess their human-specific cardioprotection in vitro, we developed a human heart-on-a-chip (hHOC) IRI assay using human stem cell-derived, engineered cardiac tissues. We found that EEVs alleviated cardiac cell death as well as the loss in contractile capacity during and after simulated IRI, in an uptake- and dose-dependent manner. Moreover, we found that EEVs increased the respiratory capacity of normoxic cardiomyocytes. These results suggest that vascular EEVs rescue hlCTs exposed to IRI possibly by supplementing injured myocytes with cargo that supports multiple metabolic and salvage pathways, and therefore may serve as a multi-targeted therapy for IRI.

1 **Introduction**

2 Each year, over half a million Americans suffer from myocardial infarction (MI) (1). The most
3 effective therapeutic intervention requires timely and complete restoration of arterial blood flow
4 to the affected area (2). However, this process may induce further cardiomyocyte death and
5 contribute to the final infarct size (3), the primary determinant of left ventricular recovery
6 prospects and potential of heart failure (4). These conjugated events, termed ischemia–reperfusion
7 injury (IRI), eventually escalate to the development of ischemic heart disease (2, 4, 5). Thus,
8 additional cardioprotective strategies are needed (6). The cellular response to IRI involves multiple
9 pathophysiological mechanisms, such as calcium and proton overload, oxidative stress,
10 endoplasmic reticulum stress, mitochondrial dysfunction, activation of apoptotic pathways (5), and
11 myocardial hibernation (7). This complex set of processes impedes the development of effective
12 therapies. Additionally, patients with MI often suffer other comorbidities, which affects the
13 efficacy of treatment. Therefore, due to the complexity of the disease, it is suggested that optimal
14 cardioprotection may require synergistic, multi-targeted therapy (8). Further, the species-
15 dependency of the response to IRI (5, 9) renders it difficult to translate preclinical findings to
16 clinical protocols, imposing a pressing need for developing human-specific preclinical assays.

17 Several pharmacological, mechanical, and cell-based therapies have been investigated in
18 recent years. Ischemic postconditioning, involving short cycles of ischemia and reperfusion
19 immediately after reflow, has been shown to exert a protective effect (2), while tissue
20 revascularization approaches (10, 11) salvage viable myocardium but fail to restore apoptotic
21 tissue. Moreover, pharmacological interventions that target isolated molecules or signaling
22 pathways have shown unsatisfactory results. For example, calcium channel blockers, antioxidants
23 and anti-inflammatory agents have been proven ineffective in reducing infarct size and improving

1 clinical metrics (12). Similarly, agents targeting mitochondria and modulation of nitric oxide
2 signaling have failed in recent clinical trials (4). Cell therapy approaches are generally safe, yet
3 their efficacy remains controversial (13). Several clinical trials have found little benefit in
4 transplantation of bone marrow-derived stem cells for treating myocardial infarction in patients
5 (14, 15). Nevertheless, recent data suggest that a broad spectrum of factors secreted by stem cells
6 of various sources may be highly therapeutic in cardiovascular disease (16, 17). Specifically, stem
7 cell-derived extracellular vesicles (EVs), particularly exosomes, are now believed to be the key
8 source of beneficial effects related to stem cells. These EVs have been shown to simultaneously
9 trigger angiogenic and anti-apoptotic processes mediated by specific miRNA cargo (18-23).
10 Interestingly, it has recently been demonstrated that naïve, non-stem cell exosomes alleviated
11 cardiomyocyte death after IRI. This has been suggested to occur via activation of the membrane
12 receptor-dependent ERK1/2 pathway (24, 25). In view of this evidence, it is unclear whether these
13 EVs exert their paracrine effect by binding to membrane receptors that initiate intracellular
14 signaling cascades or by directly delivering RNA and protein cargo across tissues and organs.

15 Vascular endothelial cells, which are abundant in perfused tissues, act as ‘first responders’
16 to hypoxic stress(26): They directly interface the parenchymal cells, and therefore are highly
17 involved in paracrine signaling to body tissues, including the highly vascularized heart (27).
18 Similar to other cell types (28, 29), endothelial cells secrete EVs, such as exosomes, which convey
19 messages in the form of protein and RNA cargo to recipient cells (*e.g.*, cardiomyocytes). We
20 hypothesized that endothelial cell-derived extracellular vesicles (EEVs) contain cargo that can
21 impart direct protective effects on cardiac muscle. To assess whether EEVs’ protein content could
22 play a role mediating such effects, we mapped the vascular EEVs’ proteome. Doing so, we found
23 a diverse population of over 1800 proteins with a high abundance of likely candidates for

cytoprotection in ischemia-reperfusion injury, including several proteins previously associated with ischemic preconditioning. We tested the preconditioning-like cardioprotective effect exerted by these EEVs on human tissue using a human tissue heart-on-chip (hHOC) IRI assay. In the hHOC model, human cardiac myocytes (hCMs) are assembled into a layer of ventricular-like muscle, and tissue contractile function is continuously monitored during and after ischemic conditions using embedded sensors (28, 29). We found that EEVs derived from normoxic and hypoxic endothelial cell cultures increased respiratory capacity in normoxic cardiomyocytes and limited myocardial cell death and loss of contractile function associated with IRI. Proteomics analysis demonstrated that EEV treatment resulted in a shift of the protein landscape of myocytes from an IR-injured to non-injured profile. Furthermore, we observed that the EEV-mediated cardioprotective effect was dose-dependent and required internalization. In light of the reported findings, we propose that EEVs protect human laminar cardiac tissue (hlCT) from IRI in part by supplementing injured myocytes with proteins that support multiple metabolic and salvage pathways—thus paving a way for new therapeutic approaches. Simultaneously, this study demonstrates the use of the hHOC system for modeling IRI in vitro, with real time data for assessing therapeutic strategies and their dynamic effects on IRI.

Results

Endothelium-derived extracellular vesicles isolation and characterization

Endothelial-derived EVs may directly influence cardiac muscle viability and contractile function in IRI (Fig. 1A). To elucidate such effects, we produced EVs from human umbilical vein endothelial cell (HUVEC) culture and designed an in vitro system whereby contractile function of hCM-derived laminar cardiac tissues is measured continuously in a controlled environment (Fig. 1B). To test whether EV secretion from endothelial cells is modulated in response to hypoxia, we

isolated EVs from normoxic (21% O₂) and hypoxic (1% O₂) HUVEC cultures (Fig. 1C). Transmission electron microscopy (TEM) micrographs support the presence of EVs in the acquired pellet (Fig. 1D). Proteomics analysis further confirmed that the isolated fraction contained subpopulations of exosomes by showing the abundance of known exosomal markers (30), such as tetraspanins (CD9, CD63, CD81), HSP70, RAB proteins, and others (Fig. 1E) (31). The average vesicle diameter was 163±10 nm and 151±7 nm for normoxic and hypoxic EEVs, respectively; both within the upper size range for exosomes (Fig. 1F). EEV yield per isolation (25-30 T175 culture flasks) was in the range of 10⁹-10¹¹, depending on HUVEC culturing conditions. The EEV production rate was 6.5±1.7-times higher for hypoxic compared to normoxic EEVs (Fig. 1G). Overall, our data suggest that both normoxic and hypoxic endothelial cultures produce heterogeneous populations of EVs (*e.g.*, exosomes and microvesicles) that can be isolated using differential ultracentrifugation.

Mass spectrometry assessment of EEV protein content

To characterize the putative EV-mediated signaling between endothelial cells and cardiomyocytes, we mapped and analyzed the endothelial cell-derived EV protein cargo. After removing common protein contaminants, including some of bovine origin (table S1), we identified >1,800 proteins in both the normoxic and hypoxic EEV samples (data file S1). The number of identified proteins is consistent with recent proteomic studies of other EVs' proteomes (32). To assess the proteins' biological functions and potential physiological relevance to cardioprotection, we used a battery of bioinformatics analyses. The proteomaps (Fig. 2A) exhibited a high degree of diversity in the biological functions of the EEVs proteomes, particularly with respect to signaling intermediates, metabolic proteins, and protein expression/protein folding factors. A strikingly high abundance of ribosomal and protein folding-related proteins was presented in the maps. This result suggests that

EEVs may enhance protein synthesis and regulation of protein structure, as well as play a role in translation, transcription, DNA repair, regulation of cell growth, and apoptosis (33-35). Ribosomal proteins have previously been reported in EV fractions isolated using ultracentrifugation (36) and sucrose gradient techniques (37, 38). Yet, these were absent in fractions isolated using size exclusion chromatography (39) or high-resolution density gradients (31). Therefore, EV isolation methods have fundamental influence on content.

We then compared protein content between EEVs derived from normoxic and hypoxic conditions. Strong positive correlations were found between the technical replicates within these sample groups, and moderately positive correlations between the normoxic and hypoxic samples (Fig. 2B). These correlations suggest consistency between sample runs and similarity between the normoxic and hypoxic EEVs. Comparing the protein content of normoxic and hypoxic EEV samples, we identified fewer than 100 proteins that were significantly ($p \leq 0.05$) differentially expressed between normoxic and hypoxic conditions (fig. S1A). The majority of differentially expressed proteins were related to genetic information processing. Smaller subsets of the differentially expressed proteins were related to metabolism, cellular processes, and environmental information processing (fig. S1B).

Subsequently, to identify the cellular compartments (Fig. 2C) and biological processes (Fig. 2D) that were significantly ($P \leq 0.05$) overrepresented in the data set, we used gene ontology enrichment analysis. Overrepresented clusters included cytoskeleton, endoplasmic reticulum, membrane and nuclear proteins in the compartment cluster, and metabolism, transport, protein folding and cellular response to stress and ROS in the biological processes cluster (table S2, A and B). Metabolic proteins were related to glucose metabolism, such as glycolytic enzymes and lactate dehydrogenase (LDH), as well as fatty acid metabolism and cellular respiration.

1 To investigate the direct effect of the EEVs on the hCMs, we mapped and analyzed the
2 protein content of normoxic EEV-loaded vs. untreated hCMs. A proteomap representing the
3 significantly ($P \leq 0.05$) differentially expressed proteins (DEPs, data file S2) is presented in Fig.
4 3A. The signature of this proteomap is similar to the one representing the EEVs cargo (Fig. 2A).
5 Overall, 394 significantly DEPs were detected, 183 of which were overexpressed in the EEV-
6 treated myocytes. Of the 183 overexpressed proteins, 97 proteins were enriched in EEVs (Fig. 3,
7 B and C, overlapping proteins labeled in red). Again, we used gene ontology enrichment analysis
8 to identify the cellular compartments and biological processes. Overrepresented clusters of the
9 DEPs included cytoskeleton, endoplasmic reticulum, nuclear and mitochondrial proteins (Fig. 3D),
10 as did the EEVs. Further analysis of the DEPs revealed changes in various biological processes
11 including cellular response to stress, metabolism, respiration, and energy derivation (Fig. 3E), as
12 well as translation and signal transduction (Fig. 3A). Major molecular function and biological
13 process classification of the DEPs are depicted in figs. S2 and S3.

14 Due to the observed enrichment of metabolic process and mitochondrial proteins in the
15 EEVs and in the EEV-loaded hCMs, we sought to assess the effects of EEVs on mitochondrial
16 function in hCMs. To probe this, we used an extracellular flux analyzer to measure oxygen
17 consumption rates (OCRs) (40). Our results show that baseline OCR was similar in the EEV-
18 treated and control groups (Fig. 3F). When hCMs were treated with oligomycin, an ATP synthase
19 inhibitor, the measured OCR values were still similar in the different experimental groups.
20 However, when depleting the mitochondrial membrane potential using carbonyl cyanide-4-
21 (trifluoromethoxy)phenylhydrazone (FCCP), a notably higher OCR was observed for EEV-treated
22 cultures, indicative of a larger spare respiratory capacity (Fig. 3, F and G). Lastly, non-
23 mitochondrial oxygen consumption was similar in the EEV-treated and control cultures (Fig. 3F),

as seen after electron transport was blocked using antimycin A and rotenone. In summary, EEVs significantly ($p=0.0195$, for normoxic and $p=0.0019$ for hypoxic EEVs) increase the spare respiratory capacity in hCMs while baseline and ATP synthesis are unaltered.

Since the EEVs and EEV-treated hCMs were also enriched in proteins related to cellular response to mitochondrial stress, we sought to measure intracellular reactive oxygen species (ROS) of hCMs in response to an oxidative stress-inducing drug. Menadione is a compound that undergoes redox cycling with formation of ROS. Measuring intracellular ROS after menadione treatment demonstrated slower increase in ROS in hCMs pre-treated with EEVs (fig. S4).

Together, our results support that EEV treatment of myocytes lead to changes in protein profile associated with various cellular components and biological processes. Metabolic process and mitochondrial proteins were particularly enriched in EEVs, resulting in EEV-treated hCMs having larger bioenergetic reserve and being slightly more resistant to ROS.

Cardioprotective proteins in EEVs

We then asked whether EEVs contained proteins that can target one or more of the pathophysiological mechanisms initiated by IRI. This process leads to cellular organelle and cytoskeletal damage, dysfunction, and ultimately cell death via multiple pathways (5). We identified seven groups of proteins related to these IRI-induced deleterious mechanisms in both normoxic and hypoxic EEV samples (Fig. 4A and table S3): (i) nine antioxidants; (ii) nine out of the ten enzymes participating in glycolysis; (iii) sixteen AMPK-related proteins; (iv) thirteen proteins involved in the protective unfolded protein response (UPR) pathway; (v) seven proteins involved in cellular redox homeostasis; (vi) nine heat shock proteins (HSPs) that are known to

1 affect multiple pathological processes and ameliorate myocardial IRI; and (vii) five proteins
2 related to calcium homeostasis.

3 In addition to proteins that may target the pathophysiological mechanisms initiated by IRI,
4 we identified a group of proteins in the EEVs that are known to be downregulated in
5 cardiomyocytes during IRI (Fig. 4B). These proteins are simultaneously known to be positively
6 affected by ischemic preconditioning treatment. Ischemic preconditioning was previously found
7 to upregulate 30 out of 64 genes that are downregulated after myocardial IRI (41). Of these, 21
8 respective proteins were found in the EEVs. The majority of these are involved in energy
9 production and conversion, as well as metabolism. In addition, proteins respective to 6 out of the
10 34 downregulated genes that were not affected by preconditioning were found in EEVs. These
11 findings suggest that EEVs may induce a cardioprotective effect partly via pathways similar to
12 those associated with ischemic preconditioning.

13 Protein-protein interaction network analysis of the protein clusters (Fig. 4C) identified a
14 high degree of connectivity between the preconditioning-associated proteins and the seven
15 identified protein groups that may target IRI pathophysiological mechanisms. Particularly, these
16 are proteins heavily involved in cellular metabolism and repair (*i.e.*, “glycolysis”, “AMPK”, and
17 “HSP” protein groups). The observed high connectivity implies synergistic activity of the different
18 clusters in modifying the cellular response to IRI, whereby multiple pathways can be targeted by
19 EEVs to induce a cardioprotective effect. Together, these EV-residing proteins may assist in
20 preservation of cellular integrity, structure, and function, as well as inhibit the onset of apoptotic
21 signaling initiated by pathological IRI (Fig. 4D).

22 *Effect of EEVs on cardiac tissue viability after simulated ischemia-reperfusion injury*

1 To test whether EEVs could be cardioprotective in hCMs during IRI, we investigated their
2 cardioprotective effect on cardiomyocytes derived from human stem cells. To simulate ischemia
3 on hCMs, we replaced the cardiac cell culture medium with glucose-deprived, lactate-containing,
4 and acidic “ischemia buffer”, followed by exposure to 1% O₂ environment. After 3 hours under
5 ischemic conditions, reperfusion was simulated by returning to cell culture medium and restoring
6 the O₂ to 21% for 1.5 hours before conducting viability measurements. Live/dead cell assays
7 showed close to 50% cell death, indicating extensive damage to the hCMs.

8 To test the hypothesis that EEVs can improve the survivability of human myocardium after
9 IRI, we treated hCMs with two equal doses of normoxic and hypoxic EEVs. One dose was given
10 3 hours prior to the onset of ischemia and the second right after 3 hours ischemia (before
11 reperfusion) (Fig. 5A). End-point quantification of the fraction of dead cells in IRI-challenged
12 engineered myocardial tissue (Fig. 5B) demonstrated a statistically significant ($p=0.0001$ for
13 normoxic and $P<0.0001$ for hypoxic EEVs) ~50% increase in tissue viability in the presence of
14 either normoxic and hypoxic EEVs (Fig. 5C). Notably, preconditioning hCMs with 10 minutes
15 ischemia and 10 minutes reperfusion increased tissue viability by ~23% relative to untreated
16 controls (fig. S5). In addition, secreted LDH, an enzyme indicating cellular damage, was lower in
17 EEV-treated tissues (Fig. 5D). The effect of EEVs on cardiac tissue viability proved to be dose-
18 dependent, as demonstrated after exposure of hCMs to increasing concentrations of normoxic or
19 hypoxic EEVs (5×10^6 , 5×10^7 , 5×10^8 and 5×10^9 EEVs/well) prior to IRI (Fig. 5E). The fraction of
20 dead cells decreased with increasing EEV concentration relative to untreated cells, for both
21 normoxic and hypoxic EEV populations. Notably, the cardioprotective effect did not reach a
22 plateau in the tested range, implying that stronger cardioprotection might be possible. However,
23 we did not increase EEV concentrations due to technical limitations of upscaling EEV production.

1 To elucidate the cellular processes involved in the EEV-mediated rescue of viability, we
2 compared the protein profiles of IRI-challenged hCMs with and without EEV treatment. We found
3 ~1350 proteins that were significantly ($P \leq 0.05$) differentially expressed in the EEV-treated
4 population (data file S3) and analyzed their cellular component and biological process
5 classifications. The proteomap was similar to that derived from the EEV samples, with DEPs
6 including metabolic proteins, and protein expression/protein folding factors (Fig. 6A). The DEPs'
7 cellular component (Fig. 6, B and C) and molecular function (Fig. 6D, fig. S6) classifications
8 showed dominant mitochondrial and catalytic activity-related proteins. This implies differences in
9 the metabolic landscape of the EEV-treated injured cells. The biological processes related to the
10 DEPs include cellular response to stress, respiration, energy derivation, glucose, and fatty acid
11 metabolism, as well as protein targeting and folding (Fig. 6E, fig. S7). Principal component
12 analysis of injured / uninjured and EEV-treated / untreated hCMs demonstrated that EEVs only
13 slightly affect the protein profile of uninjured cells, whereas the larger modifications induced by
14 IRI were partially rectified by the EEV treatment (Fig. 6F).

15 The EEV-dependent effect on hCMs' viability and protein expression after IRI may be
16 mediated by cargo delivered into the cell via endocytosis or activation of a ligand-receptor
17 response on the cell membrane without EEV internalization (24). To determine whether
18 endothelial cell-derived EVs are internalized by cardiomyocytes, we produced fluorescent EEVs
19 by labeling HUVECs with DiO, a green lipophilic tracer that is transferred to the cell membrane
20 (Fig. 6, G and H). Using time-lapse microscopy, we observed internalization of these labeled EEVs
21 by hCMs within 45 min (Fig. 6H, fig. S8, movies S1-S3). Therefore, we hypothesized that the
22 cardioprotective effect of the EEVs was mediated by post-internalization cargo release. To test
23 this hypothesis, we assessed whether inhibition of endocytosis in cardiomyocytes reduces the

EEV-induced protective effect observed during IRI. Endocytosis can occur via multiple pathways, some of which include inwards budding of the plasma membrane and formation of early endosomes – the first compartment of the endocytic pathway. The inward budding of the plasma membrane can occur in specific domains and by different mechanisms. We targeted two endocytosis routes: dynamin- and lipid raft-mediated endocytosis (Fig. 6I). Dynamin-mediated endocytosis was inhibited by using dynasore (42), which prevents dynamin from tightening around the inward budding vesicle neck (*e.g.*, clathrin-coated pit) and the formation of an endosome (43). Lipid raft-mediated endocytosis was inhibited by using filipin (44). The lipid rafts are cholesterol-rich plasma membrane subdomains that form an inward budding vesicle. Filipin prevents shuttling into the cell by cholesterol sequestration which interrupts the rafts (45). Although neither dynasore nor filipin had any direct effect on cell viability among untreated control samples, they both significantly (*P-values* detailed in data file S4) reduced the EEV-mediated cardioprotection as measured by an increased fraction of dead cells (Fig. 6J). Together, our results support the notion that EEVs interfere with the fatal IRI-associated pathophysiological pathways and mediate their cardioprotective effect by releasing their cargo within cardiomyocytes.

Effect of EEVs on cardiac tissue contractile function during ischemia-reperfusion injury

Recovery of viable tissue contractile function after IRI is a key factor in determining the outcomes of myocardial infarction and the possible development of heart failure. To determine whether EEVs aid in preservation and recovery of contractile function after IRI, we used an instrumented hHOC (28, 29) platform. The hHOC platform features engineered hLCTs on soft cantilever substrates. The tissues are guided into an anisotropic and laminar architecture that recapitulates the native myocardial muscle, through micropatterned cues on the cantilever surface (28, 29, 46,

46). In the instrumented hHOC, each cantilever further contains an embedded strain gauge (Fig. 7A and fig. S9). Upon myocardial tissue contraction, the cantilever deflects, leading to a resistance change in the embedded strain sensor that is directly proportional to tissue contractile stress (Fig. 7B). Continuous recording of the gauge resistance thus provides readout of the contractile stress of an engineered cardiac tissue, before, during, and following simulated IRI. Conditioning of hHCTs with normoxic or hypoxic EEVs had no direct effect on baseline contractile activity (Fig. 7C-D). However, upon exposing the engineered cardiac tissues to ischemia, we observed an almost immediate cessation of tissue contraction and a minor recovery of twitch stress following reperfusion (Fig. 7E, top panel). However, EEV-treated tissues continued contracting during simulated ischemia, and displayed significant ($p=0.0005$ and $p=0.0172$ for normoxic and hypoxic EEVs) recovery of twitch stress following reperfusion (Fig. 7E). While the contractile twitch stress in untreated tissues decreased to below 10% of baseline during simulated ischemia, tissue contractile stress decreased to merely $40\pm 8\%$ and $36\pm 10\%$ of baseline for cardiac tissues conditioned with normoxic and hypoxic EEVs, respectively (Fig. 7F). Furthermore, twitch stress recovery after reperfusion was $77\pm 9\%$ and $61\pm 11\%$ for normoxic and hypoxic EV-conditioned tissues respectively, whereas in untreated tissues, twitch stress recovery was only $18\pm 7\%$ of baseline (Fig. 7G).

In addition to testing the response of human cardiac tissues to the EEV treatment, we constructed neonatal rat ventricular myocytes (NRVMs) tissues in the same HOC system (fig. S10A) and exposed them to the same simulated IRI protocol and EEV treatment. Interestingly, EEVs were internalized in the NRVMs (fig. S10B), yet their protective effect on NRVM-derived tissues viability and contractility was notably smaller (fig. S10C-E).

Discussion

The key finding of this study is that vascular endothelial cell-derived extracellular vesicles exert a protective effect against myocardial IRI in human tissue. Importantly, this cardioprotective effect is manifested as a significant reduction in cell death, partial conservation of the cellular proteome, as well as preservation of tissue contractility as demonstrated using our IRI hHOC assay.

In control tissues without EEV treatment, the loss of contractile function was greater than the loss in viability. This mirrors in vivo IRI, where tissue contractile function is impaired not only due to cardiomyocyte death, but also due to ATP depletion, abnormal calcium concentrations, and damage to contractile filaments of the surviving myocytes (5). In EEV-treated tissues, both viability and contractile function were improved, and the rescue effect on contractile function was greater than the effect on viability. Proteomics analysis revealed that EEVs shifted the protein profiles of IR-injured hCMs closer to their uninjured signature, indicating that EEVs likely exert their cardioprotective effects by both increasing survivability of cardiomyocytes and preserving cellular processes, function, and integrity.

Previous studies have reported cardioprotective effects of exosomes derived from various cell sources (18-21, 24, 25), but the mechanisms of action and the origin of protective exosomes remain elusive. Vicencio *et al.* (24) reported that plasma-derived exosomes were cardioprotective both in vivo and in vitro, using adult rats and adult rat myocytes. In that study, the authors did not observe exosome uptake, and proposed that exosomal HSP70 may bind to Toll-like receptor 4 to activate the protective ERK1/2-HSP27 signaling pathway. Other studies reported uptake of stem cell-derived (21) or cardiac progenitor cell (CPC)-derived (20) protective exosomes in cardiomyocytes. Barile *et al.* (20) suggested that CPC-derived exosomes induced cardioprotective effects in vitro and in a mouse MI model, by delivery of anti-apoptotic and pro-angiogenic

miRNAs. Similarly, Khan *et al.* (21) reported that mouse embryonic stem cell-derived exosomes improved ejection function and reduced infarct size and apoptosis in a mouse model of myocardial infarction. Notably, they reported that the effect involved neovascularization and expansion of cardiac progenitor cells mediated by the embryonic stem cell-specific miRNA-294. In both studies, the protective effect could not be recapitulated using fibroblast-derived exosomes. Here, we used endothelial cells as a source for EVs. Primary human endothelial cells are relatively easy to obtain, expand, and culture, providing a convenient source for this therapeutic approach. Our study shows that: (i) the protective effect doesn't require stem cells or stem cells derivatives (EVs), at the site of action; (ii) the effect is caused by an intracellular mechanism, as it requires uptake; and (iii) the protective effect is associated with both increased survivability and improved contractile function of cardiomyocytes by a complex mechanism. Notably, neither filipin nor dynasore fully suppressed the EEV-mediated rescue of viability. This result implies that EEV uptake by hCMs is mediated by multiple routes of endocytosis. Since the EEVs contain diverse protein and nucleic acids cargo, it is impossible to delineate, at this point, the differential effects of the various proteins or RNA cargo. However, a recent study by Perez-Boza *et al.* (48) mapped the RNA landscape of HUVEC-derived EVs isolated by ultracentrifugation and compared it with the RNA content of the parent cells. We examined this dataset for specific miRNA previously associated with cardioprotection (table S4). Notably, miR-146a, miR-210 and miR-294, which have been linked to cell survival (19-21), were not detected in the EEVs. On the other hand, pro-vasculogenic miR-132 (20) and the pro-contractile miR-21-5p (49) were not in higher abundance with respect to the parent cells. Therefore, the beneficial effects of the EEVs could be mediated in part by miR-132 and miR-21-5p, or other mRNA content. However, as our *in-vitro* system consisted of only hCMs, pro-vasculogenic and anti-inflammatory effects would not contribute to the cardioprotection

1 observed here. Moreover, we observed a range of effects beyond increased survival, including
2 improved function during and following ischemia, increased cellular respiratory reserve, and
3 slightly decreased ROS production in menadione-challenged cells. This implies that multiple
4 factors likely contribute to the net effect.

5 Analysis of EEV proteome supports that protein content could play a key role in such a
6 multifaceted mechanism (table S3). A multitude of the identified EEV proteins are involved in
7 cellular homeostasis and preservation. Antioxidants, high abundance of heat shock proteins (50),
8 UPR intermediates and ER-resident chaperones (51, 52), as well as ribosomal proteins such as
9 Rps3a that regulate apoptosis (34, 35, 53), were harbored in the EEVs, all of which are known to
10 increase cell survivability. Furthermore, we found several proteins that support recovery of
11 contractile structures and metabolic function, such as glycolytic enzymes and AMPK signaling
12 intermediates which can collectively increase glycolytic activity (54, 55) and ATP production in
13 the post-ischemic myocardium (56-59). Interestingly, we found that EEV treatment resulted in
14 modulation of the expression of proteins related to various metabolic and energy derivation
15 pathways, as well as increased reserve respiratory capacity, which has been shown to be regulated
16 by AMPK (60). This increased reserve respiratory capacity can be utilized by the cells in cases of
17 metabolic stress and has been shown to increase their survival by adapting to metabolic stress (60,
18 61). Moreover, heat shock proteins can improve the repair of structural proteins after ischemia-
19 induced cytoskeletal damage (62) and assist in suppressing oxidative stress (63-65). Additionally,
20 antioxidants may mitigate oxidative stress and prevent the associated damage to structural proteins
21 and organelles. Consistent with these findings, the protein expression profile of injured hCMs
22 treated with EEVs differ from the untreated injured cells and is more similar to uninjured
23 cardiomyocytes.

1 In addition to significant post-IRI recovery of contractile function, we also observed that
2 the electrically paced, EEV-treated tissues maintained contractile function during the prolonged
3 ischemic phase of IRI, whereas control tissues ceased contracting almost immediately. This is in
4 accordance with the observed increase in maximum respiratory capacity for EEV-treated hCMs.
5 Interestingly, EEVs contained high abundance of LDH and other proteins involved in lactate
6 metabolism. These proteins can promote the conversion of lactate to pyruvate and provide an
7 anaerobic metabolic substrate. Consistently, upregulation of proteins involved in pyruvate
8 metabolism, gluconeogenesis and galactose metabolism pathways were observed in ischemia-
9 reperfusion injured hCMs that were pre-treated with EEVs, as compared to injured, untreated cells.
10 To note, in the hibernating myocardial tissue that can sustain ischemia, lactate can be used as a
11 metabolic substrate and enable contraction in response to inotropic stimulus (7, 66).

12 We found that normoxic and hypoxic EEVs had similar protein content. Major DEPs
13 consisted only 5% of the entire cargo, which were related to genetic information processing and
14 metabolism. However, the functional difference in the two EEV populations is still not clear since
15 they had similar effects on cardioprotection, basal respiration, ATP production, spare respiratory
16 capacity, and non-mitochondrial respiration. The extrapolated EEV production rate was higher
17 under hypoxic conditions, in accordance with prior findings (67, 68). It is unknown whether EEVs
18 play a role as cardioprotective agents against IRI *in vivo*, however this observation, together with
19 the observed dose dependency, suggests that an innate signal modulation by quantity could be
20 possible.

21 In this study, we used a recently published human ‘Heart-on-a-Chip’ *in-vitro* IRI assay (28,
22 29) to examine the effects of endothelial cell-derived EVs on the outcome of myocardial IRI.
23 Embedded sensors in the hHOC allowed for continuous monitoring of tissue contractile function

1 from within a closed environment. This enabled us to observe the isolated, dynamic functional
2 response of hLCTs to IRI and concomitant EEV treatment in vitro. Although the engineered hLCTs
3 in the hHOC system generated contractile activity and responded to simulated IRI as expected,
4 there are also inherent limitations. These include: (i) the partial immaturity of stem cell derived
5 hCMs; (ii) the homogeneity of engineered myocardial tissues (*i.e.*, only hCMs); and (iii) the
6 configuration of hCMs as two-dimensional monolayers, which do not fully recapitulate the three-
7 dimensional, multi-cellular adult heart. Nevertheless, we previously showed that recapitulating the
8 extracellular matrix (ECM) and anisotropy of the native myocardium promote tissue maturation
9 as measured by structural organization, contractile performance and sarcomeric gene expression
10 profiles (46). We have also recently shown that such tissue models can be used for rapid screening
11 of pharmacological agents and confirmed that they respond appropriately to a wide range of known
12 cardiac and cardiotoxic compounds (29). Moreover, in this study, we showcase the advantage of
13 using such in vitro systems by demonstrating that the cardioprotective effect exerted by EEVs on
14 laminar hCMs could not be recapitulated with NRVM-derived tissues. This approach may enable
15 us to choose an appropriate in vivo model for further testing of the suggested therapeutic approach.

16 Among current interventions to reduce ischemic damage, ischemic preconditioning is the
17 most efficient intervention in reducing infarct size and is known to improve cell survival and
18 recovery of mechanical function by activating multiple pathways (69, 70). Preconditioning has
19 been shown to decrease the fraction of dead cells by up to 50% and improve recovery of contractile
20 force two-fold, following only 90 minutes of ischemia and reperfusion in human in-vitro/ex-vivo
21 models (69, 70). In comparison, we found that the fraction of dead cells was similarly reduced by
22 50% and that recovery of contraction was approximately four times higher in EEV-treated tissues
23 than in control tissues following 3 hours of simulated ischemia and 90 minutes reperfusion.

1 Additionally, preconditioning hlCTs with 10 minutes ischemia and 10 minutes reperfusion,
2 followed by prolonged (3 hours) ischemia and reperfusion (90 minutes) resulted in lesser viability
3 compared to EEV treated, injured hlCTs. This comparison suggests that the EEV-induced
4 cardioprotective effect could be at least as effective as the protective effect induced by
5 preconditioning.

6 Our findings indicate that EEVs could constitute a multi-target therapy for IRI, but several
7 challenges are apparent for translating EVs into a clinically relevant therapy. For instance, the cell
8 source used here for EEV derivation was venous endothelial cells. Although they produce an
9 apparent cardioprotective effect, other cell types, for example, arterial endothelial cells, may be
10 more relevant as a cell source for EVs. Moreover, the three-hour gap between EEV treatment and
11 IRI induction applied in this study is clearly of limited relevancy for a clinical setting. Thus, an
12 assessment of the therapeutic effect of EEVs when administered before or immediately after
13 reperfusion, is necessary for clinical translation. Similarly, studying the most effective time
14 window for a cardioprotective effect will be necessary for translation of the findings presented
15 herein. A question remains regarding the duration of EEV cargo-induced effects, and how they
16 vary with time after treatment. As a further obstacle for clinical translation, traditional cell culture
17 may not be sufficiently scalable for high throughput production of EVs. Finally, issues such as
18 dosing efficiency, clearance, targeted delivery and EVs cargo optimization to increase therapeutic
19 efficiency, must still be evaluated. To optimize the treatment an appropriate in vivo model should
20 be designed, which could be facilitated by the HOC system as demonstrated here with both human-
21 and NRVM-derived tissues. In that sense, the present results may represent a starting point for
22 production of therapeutic EV-mimetics.

Materials and Methods

Study design

The aims of this study were to characterize the protein cargo of human vascular EEVs, to identify lead cardioactive proteins, and assess the effect of EEVs on hICTs exposed to IRI. Based on analysis of EEV protein contents, we hypothesized that these EEVs are cardioprotective against IRI. We designed a hHOC system to test the effects of EEVs on hICT contractile function and used hCMs cultures to test the effects of EEVs on myocyte respiration, viability after IRI, and EEV uptake. Cardiac cultures or tissues in hHOC systems were randomly assigned for the various treatment groups in all the assays. The investigators were not blinded on any of the reported experiments. No sample size calculations were performed. Standard statistical methods were used to determine significance, as detailed in data file S4. We generally assumed Normal distribution with the exception of OCR data. No data were excluded unless apparent failure was observed in the following cases: (i) breakage of HOC strain sensor; (ii) cardiac tissue delamination from HOC substrate; or (iii) bacterial infections. No outliers were removed.

Experiments with hCMs in cultures or in HOC systems had $N \geq 3$ biological replicates for each group. Technical replicates were not considered as independent samples, thus multiple technical replicates of the same one biological sample were counted as $N=1$. Experiments were repeated multiple times in multiple occasions. For proteomics data, EEVs proteomics were derived from 4 samples of normoxic EEVs and 2 samples of hypoxic EEVs. Proteomic analyses of cardiomyocytes were derived from 3 samples for each treatment group, except for the untreated, uninjured control group (2 samples).

Extracellular vesicles isolation

EVs were isolated by ultracentrifugation, as previously published (71). Briefly, 12 h after passage 5 or 6, HUVECs were washed once with warm PBS and the standard endothelial growth medium was replaced with EGM containing 2% exosome-depleted fetal bovine serum (Invitrogen, Carlsbad, CA, USA). After 48 h, culture medium was collected for “normoxic EVs” isolation. Fresh exosome-free medium was then added to the flasks, and cells were then subjected to 3 h of hypoxia (1% O₂). Then, culture medium was collected for “hypoxic EVs” isolation. The culture medium was divided into 50 mL conical tubes and centrifuged at 300 g, 4°C, for 10 min. The supernatant was collected and centrifuged at 2000 g, 4°C for 20 min. Supernatant was collected and centrifuged at 10,000 g, 4°C, for 30 min. Finally, the supernatant was placed in thin wall, Ultra-Clear, 38.5 mL tubes and centrifuged at 100,000 g, 4°C, for 70 min, in an XPN-80 Beckman Coulter ultracentrifuge, equipped with an SW 32 Ti rotor (Beckman Coulter). The supernatant was discarded, and the pellet in each of the tubes was re-suspended in sterile PBS. All the “normoxic EVs” suspensions were pooled in a single tube, and the “hypoxic EVs” suspensions in another. Then, a second ultracentrifugation cycle was carried out under the same conditions. The EVs pellet was then re-suspended in 500 µL PBS, or in 150 µL lysis buffer (Covaris, Protein extraction buffer TP), if the samples were designated for proteins analysis. Particle Analysis and concentration assessment using NanoSight (NS300, Malvern) were performed with 2 µL of each sample. Samples were stored in -80°C until use or kept on ice if brought to protein analysis or used on the same day.

Extracellular vesicles labeling

EV-producing endothelial cells were initially labeled using a lipophilic tracer prior to the last passage, before being placed in the exosome-free, serum-enriched medium. Staining medium was prepared by adding 5 µL DiO or DiD stock solution (Vybrant Cell-Labeling Solutions, Life

Technologies) per 1 mL of growth medium. Culture medium was drained from T175 flasks containing confluent, adherent HUVECs at P4 or P5. The cells in each flask were then incubated with 3 mL of the staining solution, for 15 min, at 37°C. After incubation, the staining solution was drained, followed by washing cells twice with growth medium for 10 min each. After the second wash, cells were dissociated and passaged 1:3. The culture medium was then switched to growth medium supplemented with 2% exosome-depleted fetal bovine serum after 24 h. Culture media was collected for EEV isolation after 48 h, as described above. Following EEV isolation, 20 µL of labeled and non-labeled EEV suspensions were added to a black 384-well plate and loaded into a plate reader for fluorescence intensity measurement.

Simulated ischemia reperfusion injury (IRI)

To simulate IRI, cardiomyocytes were exposed to 3 h ischemic conditions and 1.5 h under reperfusion treatment. Ischemia was simulated by switching the complete culture medium to a Tyrode's-based "ischemia buffer" with (mM): KCl 4, NaCl 115, CaCl 1.8, HEPES 5, MgCl₂ 1, NaH₂PO₄ 0.33, sodium lactate 20, with no glucose, and pH 6.4. O₂ was set to 1%. For reperfusion, O₂ was increased back to 21% and medium was changed back to complete culture medium, pH 7.4. To test EEVss for a possible cardioprotective effect, cardiomyocytes were incubated with 10⁹ EEVs/well ("normoxic" or "hypoxic"), 3 h prior to onset of ischemia, with an additional, identical dose of EEVs at the onset of simulated ischemia, with the "ischemia buffer". Reperfusion was simulated by switching the "ischemia buffer" back to complete culture medium at 21% O₂ for 1.5 h. Finally, after reperfusion, media were collected for LDH measurement (Cytotox 96, Promega), and viability assays were performed. Functional contractile measurements were conducted throughout the simulated IRI.

Ischemic preconditioning

To assess and compare the rescue mediated by preconditioning compared to EEV treatment, we conducted the IRI protocol, as described above, with hCMs that were exposed to 10 minutes ischemia and 10 minutes reperfusion prior to the prolonged ischemia. hCMs were pre-plated, seeded in well plates and cultured for 6 days, as described above. On day 6, the cells were washed 3 times with warm, sterile PBS and 'ischemia buffer' (described above) was added to the wells. The well plates were then transferred to a 1% oxygen incubator for 10 minutes. After 10 minutes of simulated ischemia, the 'ischemia buffer' was removed from the wells and replaced by the normal culture media. The well-plates were then transferred to an incubator with 21% oxygen for 10 minutes, after which the prolonged ischemia and reperfusion protocol was implemented. Live / Dead assay was performed right after the 1.5 h reperfusion period. In the same experiment we used a second well plate with hCMs that were not preconditioned as a control.

Heart-on-chip real-time contractility assays

The HOC systems, multi-well devices with instrumented muscular thin film cantilever substrates, were fabricated as previously described (29). Briefly, multilayered Polydimethylsiloxane (PDMS) films (21-23 μm) were cast onto glass substrates using a spin-coating technique. An embedded flexible titanium-gold thin film (24 nm) strain gauge was deposited using an E-beam evaporator (Denton) and a custom-made shadow mask. Ridges (5 μm -tall and 4 μm -wide, spaced by 30 μm grooves) guide the formation of anisotropic cardiac thin film tissues. Cantilevers sized 3.2 mm x 4.2 mm were defined using a CO₂ laser (Epilog). A Poly(N-isopropylacrylamide) film ensured that cantilevers could detach from glass substrates when cooled briefly to room temperature. After UVO treatment and FN incubation, hCMs were seeded on hHOC devices as described above, and cultured for 6 days prior to IRI experiments. Real-time contractility data was recorded using a

multichannel DMM system (Keithley 3706a) and analyzed using a custom Matlab (Mathworks inc.) code as previously described (29).

Extracellular vesicles uptake assays

EV internalization by cardiomyocytes was visualized by time-lapse confocal microscopy. hCMs were cultured as described above and seeded onto circular 18 mm coverslips, which are coated with micromolded PDMS. After 5 days in culture, the plasma membrane and nuclei were labeled using CellMask Deep Red Plasma Membrane Stain (Life Technologies) and Hoechst (Life Technologies), respectively, according to the products' recommended manuals. Following staining, the coverslip was transferred to an imaging chamber filled with 1 mL Live Cell Imaging Buffer (Life Technologies). The microscope (Olympus IX83) was set to perform confocal imaging at several positions along the coverslip, over a period of 3 h, using a 60× objective (Olympus). Immediately after the first time point at which images were acquired, 10⁹ DiO-labeled EEVs were added to the well, and the imaging continued. The acquired images were processed using ImageJ 2.0.0 and Imaris.

Uptake inhibition assay

Clathrin-mediated endocytosis was inhibited by administration of 80 µM dynasore (Sigma Aldrich) 30 min before adding EEVs to the cardiac tissue culture wells (72). The lipid raft/caveolae-related endocytic pathway was disrupted by administration of 1 µg/mL filipin (Sigma Aldrich), 30 min prior to addition of EEVs (73). To test the effects of EEVs uptake inhibition on the EEV-mediated cardioprotection, IRI was simulated after a 3 h incubation with “normoxic” and

“hypoxic” EEVs under the inhibited endocytosis and control conditions. Following IRI, tissue viability was quantified by performing the viability assay described above.

Preparation of hCMs for proteomics analysis

Human stem cell-derived cardiomyocytes were thawed, pre-plated, cultured, and dissociated as described above. 5M hCMs were re-seeded in 8 wells in a density of 10^5 cells/cm², and cultured for 6 days. On day 6, the media was changed for all the wells. Half of the wells were treated with 3×10^{10} EEVs (5×10^9 EEVs per 10^5 cells, as used for the rest of the experiments). The other half were treated with vehicle. After 3 h, 2 of the EEV- treated and 2 vehicle- treated wells were exposed to IRI, as described above. This procedure resulted in four experimental groups: (1) hCMs, (2) hCMs+EEVs, (3) hCMs+IRI, (4) hCMs+EEVs+IRI, with two wells per group. Using 0.25% trypsin-EDTA, cells were harvested from the wells for further mass spectroscopy analysis. After centrifugation, the supernatant was removed, pellets were washed with PBS, and then centrifuged again. This was repeated for 3 times overall. After the final centrifugation step, the supernatant was removed and the pellets were transferred to -80°C.

Sample-wise Pearson correlation analysis

Pearson Product Moment Correlation (76) was used to assess the degree of the linear correlation between pairwise comparisons of the global protein expression profiles of the normoxic and hypoxic EEV samples. Pairwise comparisons were performed using the *corr()* function provided in the PANDAS version 0.19.0-2 Python package, and visualized using the Seaborn statistical data visualization Python package, version 0.7.1-1.

Statistical analysis

Data are expressed as the mean $\pm$ s.e.m for all biological replicates. Technical replicates of the same biological replicate were averaged and treated as N=1 (*i.e.*, N indicates the number of biological replicates). Statistical analyses were performed using one-way or two-way ANOVA followed by the post hoc Tukey's test with GraphPad Prism 7 software (GraphPad Software). *P*-Values less than 0.05 (*), 0.01 (**), 0.001 (***) or 0.0001 (****) were considered statistically significant. Statistical analyses and exact *P*-values for all the presented data are detailed in data file S4.

Supplementary Materials

Materials and methods.

Fig. S1. Differentially-expressed EEVs protein content in normoxic and hypoxic conditions.

Fig. S2. Molecular function GO classification of differentially expressed proteins in hCMs treated with EEVs compared with control, untreated hCMs.

Fig. S3. Biological process GO classification of differentially expressed proteins in hCMs treated with EEVs compared with control, untreated hCMs.

Fig. S4. ROS measurement in Menadione-challenged hCMs un/treated with EEVs.

Fig. S5. Preconditioning vs. EEV treatment effect on viability following IRI.

Fig. S6. Molecular function GO classification of differentially expressed proteins in injured hCMs pre-treated with EEVs compared with injured, untreated hCMs.

Fig. S7. Biological process GO classification of differentially expressed proteins in injured hCMs pre-treated with EEVs compared with injured, untreated hCMs.

Fig. S8. EEV uptake by human cardiomyocytes

Fig. S9. Heart-on-a-chip device for contractility measurements.

Fig. S10. Non-significant cardioprotective effect of human EEVs on neonate rat ventricular myocytes-based heart-on-a-chip assay.

Mov. S1. EEV uptake by human cardiomyocytes (cell 1).

Mov. S2. EEV uptake by human cardiomyocytes (cell 2).

Mov. S3. EEV uptake by human cardiomyocytes (cell 3).

Table S1. Common contaminant proteins detected in EEV samples.

Table S2. Gene Ontology terms and enrichment *P*-values for over-represented protein groups presented in figure 2C-D with corresponding color coding.

Table S3. Cardioprotective function of protein groups represented in EEVs.

Table S4. Possible cardioprotective miRNA cargo in previously published studies.

Data file S1. Total EEVs proteome raw data.

Data file S2. Differentially expressed proteins in EEV-treated vs. untreated hCMs.

Data file S3. Differentially expressed proteins in IR-injured, EEV-treated vs. injured, untreated hCMs.

Data file S4. Summary of statistical analyses and *P*-values.

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dataset.jsp?task=3421c03b031d4315a46dc8c1875ef63d. Scripts for proteomics data analysis are available at <https://github.com/spsheehy/Proteomics> and https://github.com/harvardinformatics/YadidMetal_May_2020. Scripts for contractility data analysis were previously published (26).

Figure captions:

Fig. 1. Simulating ischemia-reperfusion injury to assess cardioprotection by endothelial EVs.

(A) Schematic of the vasculature-rich heart tissue lined with endothelial cells interfacing the cardiomyocytes. This allows for paracrine signaling affecting cardiomyocytes in health and in ischemic stress conditions. EEVs can be internalized by target cells, and transfer messages by delivering proteins and RNA. (B) Schematic of heart-on-a-chip, which allows for real time measurements of contractile function, with human laminar cardiac tissues (hlCTs) to simulate IRI in the presence of EEVs. (C) Schematic diagram showing EEV isolation by a series of centrifugation steps. EVs were isolated from either normoxic (21% O₂) or hypoxic (1% O₂) HUVEC cultures. (D) TEM images of normoxic (top) and hypoxic (bottom) EVs confirm expected cup shape; scale bar= 500 nm; close-up scale bar= 200 nm. (E) Mass spectrometry analysis confirmed the extracted pellet contained EVs including exosomes, by identifying known exosomal markers. (F) Size distribution for normoxic (red) and hypoxic (blue) vesicles. (G) EEVs production rate calculated from the EEV yield divided by hours in culture before harvest, for each isolation batch.

Fig. 2. Endothelial EVs characterization. (A) Proteomaps illustrating the KEGG orthology terms and relative differences in mass abundance for the 1,820 proteins identified in normoxic (left, N=4) and hypoxic (right, N=2) EVs. (B) Sample-wise Pearson's correlation analysis

revealed a high degree of similarity between the global expression profiles for the normoxic (Norm) and hypoxic (Hypo) EV samples. Correlation coefficients between the four normoxic samples were greater than 0.9, and 0.87 for the two hypoxic samples. Gene ontology enrichment analysis identified statistically overrepresented (C) cellular localization and (D) biological function in the protein expression data set. Black vertical lines indicate P -value of 0.05.

Fig. 3. Endothelial EVs' effects on hCMs. (A) Proteomap illustrating the KEGG orthology terms and relative differences in mass abundance for the significantly (P -value <0.05) differentially expressed proteins (DEPs) in EEV-treated (N=3 samples), compared with untreated (N=2 samples), hCMs. (B) Volcano plot presenting the fold change and p -value of the DEPs. The red dots represent proteins that were significantly overexpressed in the EEV-treated myocytes and also enriched in the EEVs. (C) A Venn diagram demonstrating the amount of the DEPs in the EEV-treated myocytes, relative to the normoxic EEVs' proteome, and the overlap. (D) Cellular localization in the protein expression data set. The numbers next to the bars represent the number of significantly ($P \leq 0.05$) enriched proteins related to each of the depicted cellular compartments. (E) Dominant biological processes (BPs) in the DEPs. Each dot represents a single protein, whereas the clusters represent BPs. The red color scale represents the individual p -value for the enrichment of each protein in the data set. The dashed horizontal line represents 0-fold change, while all the dots above the line represent overexpressed proteins. The numbers above each cluster represent the false discovery rate, namely the confidence level in the representation of the specific BP. (F) Oxygen consumption rate (OCR, mean $\pm$ s.e.m) of hCMs treated with vehicle (N=6),

normoxic (Norm) EEVs (N=6), and hypoxic (Hyp) EEVs (N=6) was measured using *Seahorse* extracellular flux analyzer, while altering mitochondrial function to delineate the different components of respiration. (G) Spare respiratory capacity (mean±s.e.m) was calculated as the difference between the maximum and baseline OCR. Significance relative to untreated control was tested with one-way ANOVA. Statistical significance was presented by * $P \leq 0.05$, ** $P \leq 0.01$. *P-values* are detailed in data file S4.

Fig. 4. Identification of cardioprotection-related proteins in normoxic and hypoxic EEVs.

(A) Heat map the log₂ mass abundance values for the groups of proteins enriched in the normoxic and hypoxic EEV expression profiles associated with IRI response, and possibly involved in cardioprotection by mitigating IRI-related pathological processes. (B) Heatmap illustrating the log₂ mass abundance values for the group of proteins enriched in the normoxic and hypoxic EEV protein expression profiles associated with preconditioning. (C) Protein-protein interaction network illustrating known interactions between the proteins comprising the groups of IRI response and preconditioning-related proteins observed in the normoxic and hypoxic protein expression profiles. (D) Schematic of potential cellular mechanisms of cardioprotection mediated by the groups of proteins discovered in the EEV samples, and the related IRI-induced pathological processes that are potentially mitigated. The different groups of proteins are represented in color coding corresponding to their representation in panel A. Orange arrows stand for pathological pathways and blue arrows indicate the counteractions.

Fig. 5. EEVs are protective in myocardial ischemia-reperfusion injury. (A) The experimental

timeline illustrating timing of EEV treatment, ischemia, and reperfusion periods. (B)

Viability was assessed at end point using an Ethidium-based live/dead assay. Representative images shown for tissues untreated (left), treated with normoxic (Norm, middle), and hypoxic (Hyp, right) EEVs (Nuclei of live cells: Cyan; dead cells: red; 100µm scale bar). (C) Fraction of dead cells after reperfusion was quantified from the live/dead assay images. >5 field of views for each sample were imaged with N=9 for control; N=5 for normoxic; N=7 for hypoxic EEV treatment. Tested with one-way ANOVA. (D) Injury after reperfusion was assessed by LDH biochemical assay. LDH readout was compared between an uninjured group to the control and EEV-treated groups following IRI, with N=6 for uninjured, N=6 for control; N=7 for normoxic; N=9 for hypoxic EEV treatment. Data is represented as mean±s.e.m and tested with one-way ANOVA. Statistical significance was presented by $^{**}(P\leq 0.01)$, $^{***}(P\leq 0.001)$. *P-values* are detailed in data file S4. (E) Fraction of dead cells following IRI as a function of EEV concentration (N=3 for each group)

Fig. 6. Endothelial EVs' effects on IR-injured hCMs. (A) Proteomap illustrating the KEGG orthology terms and relative differences in mass abundance for the significantly (P -value < 0.05) differentially expressed proteins (DEPs) in EEV treated (N=3 samples), compared with untreated (N=3 samples) IR-injured hCMs. (B) Volcano plot presenting the fold change and P -value of the DEPs. The colored dots represent proteins of the cellular components represented in panel C. (C) Cellular localization in the protein expression data set. The numbers next to the bars represent the number of significantly enriched proteins related to each of the depicted cellular compartments. (D) Molecular function in the protein expression data set shows modification of catalytic activity and binding. (E) Dominant

biological processes (BPs) in the DEPs. Each dot represents a single protein, whereas the clusters represent BPs. The red color scale represents the individual *P*-value for each protein in the data set. The dashed horizontal line represents 0-fold change, while all the dots above the line represent overexpressed proteins. The numbers above each cluster represent the false discovery rate, namely the confidence level in the representation of the specific BP. **(F)** Two-dimensional principle component analysis of four treatment groups: Control – only hCMs (N=2), normoxic EEV-treated hCMs (Norm EEVs, N=3), IR-injured hCMs (IRI, N=3), and normoxic EEV-treated + IR-injured hCMs (Norm EEVs + IRI, N=3). **(G)** Schematic of production of DiO-labeled EEVs by prior labeling of cultured HUVECs' plasma membranes. DiO-labeled EEVs could be detected using confocal microscopy. **(H)** XY and reconstructed YZ planes are presented 45 minutes after adding EEVs, showing internalization of the labeled EEVs (20 μ m scale bars). Nuclei: blue; plasma membrane of cardiomyocytes: magenta; EEVs: green. **(I)** Schematic of two strategies to inhibit endocytosis in cardiomyocytes. Dynasore (left) inhibits dynamin, and Filipin (right) interrupts the lipid rafts. **(J)** Fraction of dead cells following IRI in the different treatment groups (9 experiment groups, N=4 for each group). Data is represented as mean $\pm$ s.e.m, and tested with two-way ANOVA. Statistical significance was presented by $^{*}(P\leq 0.05)$, $^{**}(P\leq 0.01)$, $^{***}(P\leq 0.001)$, $^{****}(P\leq 0.0001)$. *P-values* are detailed in data file S4.

Fig. 7. HUVEC-derived EVs rescue contractile function during IRI. **(A)** A representative image of hCM-derived tissue in the HOC system. Left image shows immunostain for nuclei (blue) and sarcomeric α -actinin (gray). Middle image shows an optical microscopy

1 tissue culture image (100 μm scale bars). On the right, a single PDMS cantilever with an
 2 embedded strain gauge is depicted (2 mm scale bar). As the hICT contracts the cantilever
 3 bends, stretching the strain gauge and leading to increased resistance. Two different
 4 bending positions are overlaid to demonstrate the cantilever's motion. The relative
 5 resistance changes ($\Delta\Omega$) are proportional to the generated twitch stress (σ). **(B)** An example
 6 trace showing measured twitch stress. **(C)** Example traces of contractile stress before and
 7 after 3-hours of EEV incubation in untreated, normoxic, and hypoxic EEV-treated tissues.
 8 The contractile stress was normalized to average amplitude before treatment. **(D)** Mean
 9 ratio between average stress before treatment to average stress after a 3-hour EEV
 10 incubation period, in control (N=6), normoxic (N=8), and hypoxic (N=5) EEV-treated
 11 tissues. No significant differences were found (one-way ANOVA). **(E)** Twitch stress
 12 amplitude normalized to baseline values throughout the simulated IRI. The bold traces
 13 present the average values over all the tested samples and shaded areas represent the SEM.
 14 **(F)** Average twitch stress during ischemia relative to baseline. **(G)** Average twitch stress
 15 recovery relative to baseline for the three experimental groups. Error bars represent s.e.m.
 16 N=6 for control; N=8 for normoxic; and N=5 for hypoxic EEV-treated groups. Tested with
 17 one-way ANOVA. There was no significant difference between the two groups treated
 18 with EEVs. Statistical significance was presented by $*(P\leq 0.05)$, $**(P\leq 0.01)$,
 19 $*** (P\leq 0.001)$. *P-values* are detailed in data file S4.

Figures

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