

## BIOENGINEERING

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

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Extracellular vesicles (EVs) derived from various stem cell sources induce cardioprotective effects during ischemia-reperfusion injury (IRI). These have been attributed mainly to the antiapoptotic, proangiogenic, microRNA (miRNA) cargo within the stem cell-derived EVs. However, the mechanisms of 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 has 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 (hICTs) 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 EEV treatment. To assess their human-specific cardioprotection in vitro, we developed a human heart-on-a-chip 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 hICTs exposed to IRI possibly by supplementing injured myocytes with cargo that supports multiple metabolic and salvage pathways and therefore may serve as a multitargeted therapy for IRI.

## INTRODUCTION

Each year, over half a million Americans suffer from myocardial infarction (MI) (1). The most effective therapeutic intervention requires timely and complete restoration of arterial blood flow to the affected area (2). However, this process may induce further cardiomyocyte death and contribute to the final infarct size (3), the primary determinant of left ventricular recovery prospects and potential of heart failure (4). These conjugated events, termed ischemia-reperfusion injury (IRI), eventually escalate to the development of ischemic heart disease (2, 4, 5). Thus, additional cardioprotective strategies are needed (6). The cellular response to IRI involves multiple pathophysiological mechanisms, such as calcium and proton overload, oxidative stress, endoplasmic reticulum (ER) stress, mitochondrial dysfunction, activation of apoptotic pathways (5), and myocardial hibernation (7). This complex set of processes impedes the development of effective therapies. In addition, patients with MI often suffer other comorbidities, which affect the efficacy of treatment. Therefore, because of the complexity of the

disease, it is suggested that optimal cardioprotection may require synergistic, multitargeted therapy (8). Furthermore, the species dependency of the response to IRI (5, 9) renders it difficult to translate preclinical findings to clinical protocols, imposing a pressing need for developing human-specific preclinical assays.

Several pharmacological, mechanical, and cell-based therapies have been investigated in recent years. Ischemic postconditioning, involving short cycles of ischemia and reperfusion immediately after reflow, has been shown to exert a protective effect (2), whereas tissue revascularization approaches (10, 11) salvage viable myocardium but fail to restore apoptotic tissue. Moreover, pharmacological interventions that target isolated molecules or signaling pathways have shown unsatisfactory results. For example, calcium channel blockers, antioxidants, and anti-inflammatory agents have proven ineffective in reducing infarct size and improving clinical metrics (12). Similarly, agents targeting mitochondria and modulation of nitric oxide signaling have failed in recent clinical trials (4). Cell therapy approaches are generally safe, yet their efficacy remains controversial (13). Several clinical trials have found little benefit in transplantation of bone marrow-derived stem cells for treating MI in patients (14, 15). Nevertheless, recent data suggest that a broad spectrum of factors secreted by stem cells of various sources may be highly therapeutic in cardiovascular disease (16, 17). Specifically, stem cell-derived extracellular vesicles (EVs), particularly exosomes, are now believed to be the key source of beneficial effects related to stem cells. These EVs have been shown to simultaneously trigger angiogenic and antiapoptotic processes mediated by specific microRNA (miRNA) cargo (18–23). It has recently been demonstrated that naïve, non-stem cell exosomes alleviated cardiomyocyte

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death after IRI. This has been suggested to occur via activation of the membrane receptor–dependent extracellular signal–regulated kinase 1/2 (ERK1/2) pathway (24, 25). In view of this evidence, it is unclear whether these EVs exert their paracrine effect by binding to membrane receptors that initiate intracellular signaling cascades or by directly delivering RNA and protein cargo across tissues and organs.

Vascular endothelial cells, which are abundant in perfused tissues, act as “first responders” to hypoxic stress (26): They directly interface the parenchymal cells and therefore are highly involved in paracrine signaling to body tissues, including the highly vascularized heart (27). Similar to other cell types (28, 29), endothelial cells secrete EVs, such as exosomes, which convey messages in the form of protein and RNA cargo to recipient cells (e.g., cardiomyocytes). We hypothesized that endothelial cell–derived EVs (EEVs) contain cargo that can impart direct protective effects on cardiac muscle. To assess whether EEVs’ protein content could play a role mediating such effects, we mapped the vascular EEVs’ proteome. Doing so, we found a diverse population of over 1800 proteins with a high abundance of likely candidates for cytoprotection in IRI, 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. Proteomic analysis demonstrated that EEV treatment resulted in a shift of the protein landscape of myocytes from an IR-injured to noninjured 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 previously unused 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 EV 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% O<sub>2</sub>) and hypoxic (1% O<sub>2</sub>) HUVEC cultures (Fig. 1C). Transmission electron microscopy (TEM) micrographs support the presence of EVs in the acquired pellet (Fig. 1D). Proteomic analysis further confirmed that the isolated fraction contained subpopulations of exosomes by showing an abundance of known exosomal markers (30), such as tetraspanins (CD9, CD63, and CD81), HSP70, RAB proteins, and

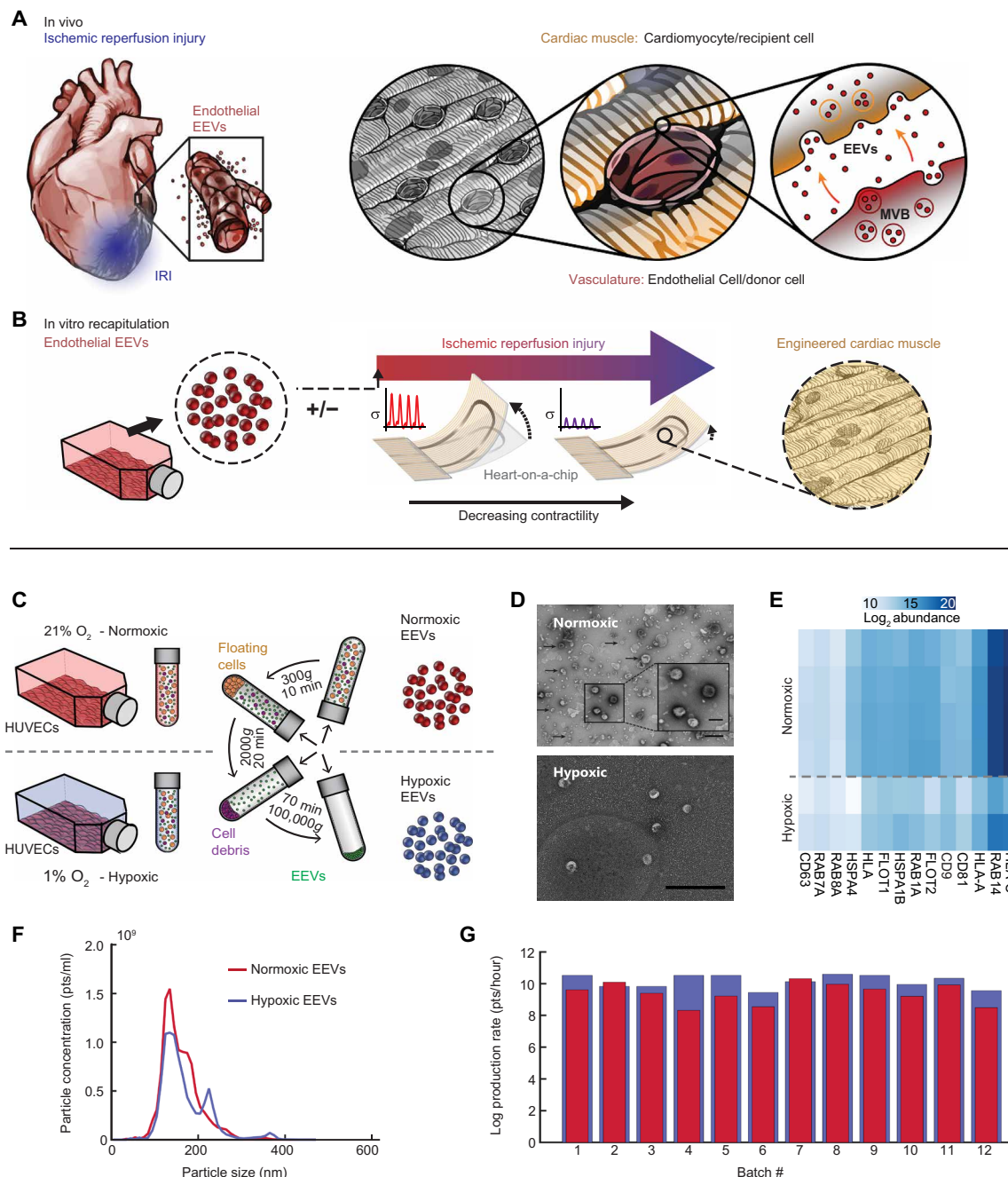
others (Fig. 1E) (31). The average vesicle diameter was  $163 \pm 10$  nm and  $151 \pm 7$  nm for normoxic and hypoxic EEVs, respectively, both within the upper size range for exosomes (Fig. 1F). EEV yield per isolation (25 to 30 T175 culture flasks) was in the range of  $10^9$  to  $10^{11}$ , depending on HUVEC culturing conditions. The EEV production rate was  $6.5 \pm 1.7$  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 >1800 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 EEV proteomes, particularly with respect to signaling intermediates, metabolic proteins, and protein expression/protein folding factors. A 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 detectable 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 were found 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 (DEPs) were related to genetic information processing. Smaller subsets of the DEPs 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 dataset, we used Gene Ontology (GO) enrichment analysis. Overrepresented clusters included cytoskeleton, ER, membrane, and nuclear proteins in the compartment cluster, and metabolism, transport, protein folding, and cellular response to stress and reactive oxygen species (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.

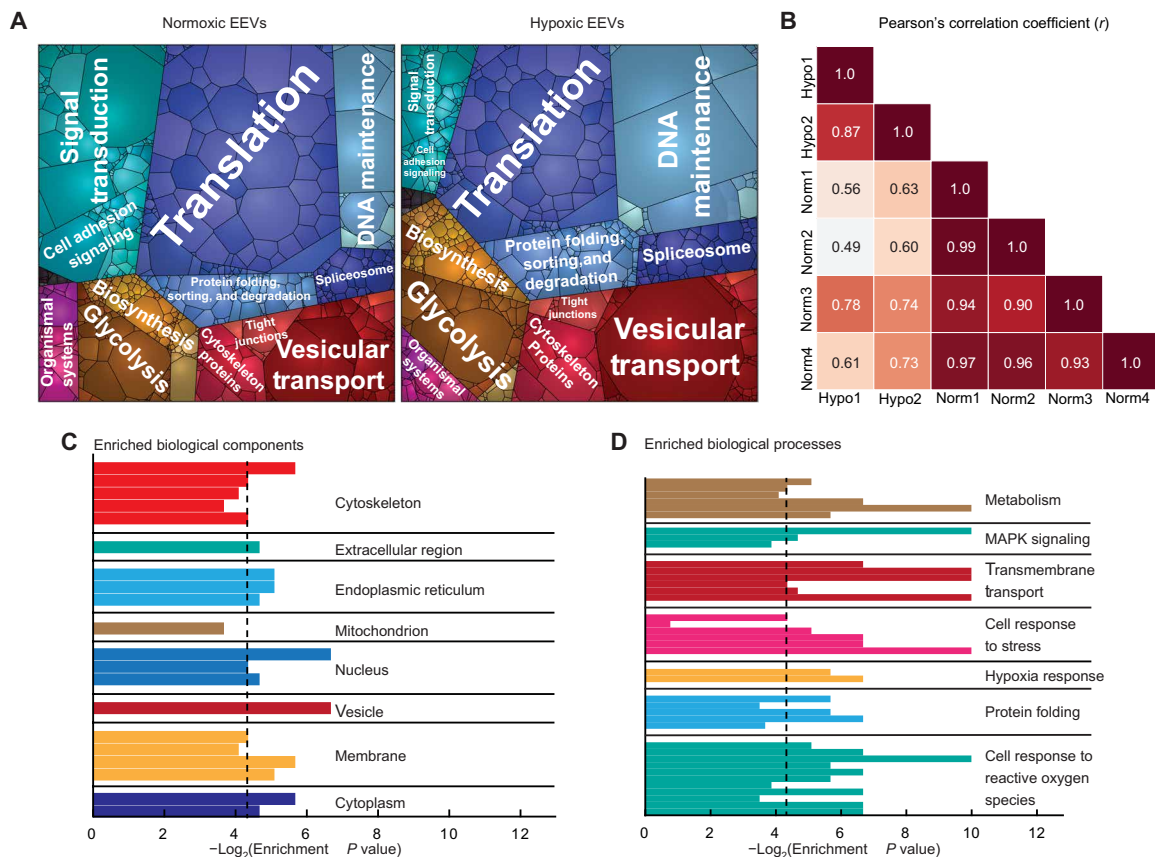


**Fig. 1. Simulating ischemia-reperfusion injury to assess cardioprotection by endothelial EVs.** (A) Schematic of the vasculature-rich heart tissue lined with endothelial cells interfacing the cardiomyocytes. This allows for paracrine signaling affecting cardiomyocytes in health and in ischemic stress conditions. EEVs, including exosomes released from multivesicular bodies (MBVs), 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% O<sub>2</sub>) or hypoxic (1% O<sub>2</sub>) 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 that the extracted pellet contained EVs including exosomes, by identifying known exosomal markers. (F) Size distribution for normoxic (red) and hypoxic (blue) vesicles. (G) EEV production rate calculated from the EEV yield divided by hours in culture before harvest for each isolation batch.

To investigate the direct effect of the EEVs on hCMs, we mapped and analyzed the protein content of normoxic EEV-loaded versus untreated hCMs. A proteomap representing the significantly ( $P \leq 0.05$ ) DEPs (data file S2) is presented in Fig. 3A. The signature

of this proteomap is similar to the one representing the EEV cargo (Fig. 2A). Overall, 394 significantly DEPs were detected, 183 of which were overexpressed in the EEV-treated myocytes. Of the 183 overexpressed proteins, 97 proteins were enriched in EEVs





**Fig. 2. Endothelial EV characterization.** (A) Proteomaps illustrating the KEGG (Kyoto Encyclopedia of Genes and Genomes) orthology terms and relative differences in mass abundance for the 1820 proteins identified in normoxic (left,  $N = 4$ ) and hypoxic (right,  $N = 2$ ) EEVs. (B) Samplewise 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. GO enrichment analysis identified statistically overrepresented (C) cellular localization and (D) biological function in the protein expression dataset. Black vertical lines indicate  $P$  value of 0.05.

(Fig. 3, B and C, overlapping proteins labeled in red). Again, we used GO enrichment analysis to identify the cellular compartments and biological processes. Overrepresented clusters of the DEPs included cytoskeleton, ER, nuclear, and mitochondrial proteins (Fig. 3D), as did the EEVs. Further analysis of the DEPs revealed changes in various biological processes including cellular response to stress, metabolism, respiration, and energy derivation (Fig. 3E), as well as translation and signal transduction (Fig. 3A). Major molecular function and biological process classification of the DEPs are depicted in figs. S2 and S3.

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

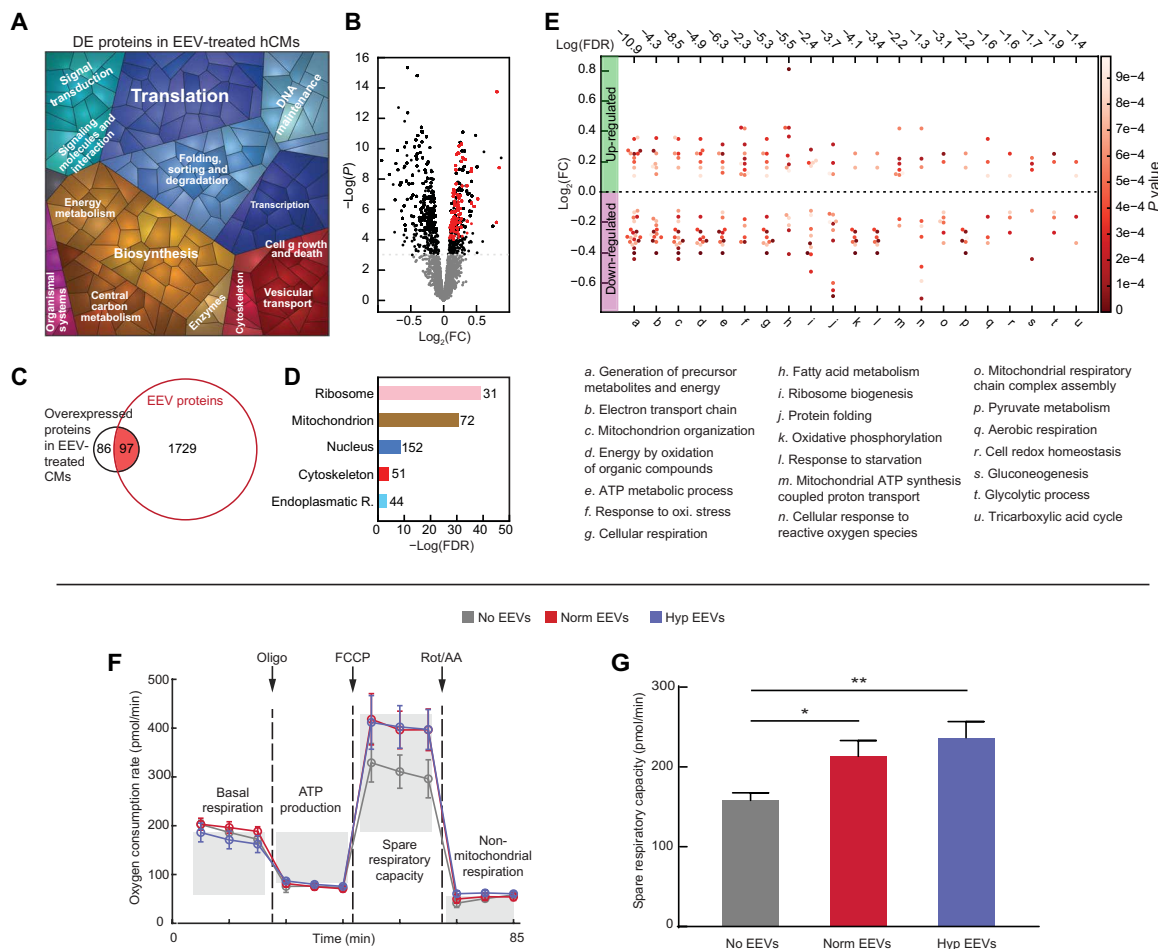
in the EEV-treated and control cultures (Fig. 3F), as seen after electron transport was blocked using antimycin A and rotenone. In summary, EEVs significantly ( $P = 0.0195$  for normoxic EEVs and  $P = 0.0019$  for hypoxic EEVs) increase the spare respiratory capacity in hCMs, whereas baseline and ATP synthesis are unaltered.

Because the EEVs and EEV-treated hCMs were also enriched in proteins related to cellular response to mitochondrial stress, we sought to measure intracellular 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 a slower increase in ROS in hCMs pretreated with EEVs (fig. S4).

Together, our results support that EEV treatment of myocytes leads 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).



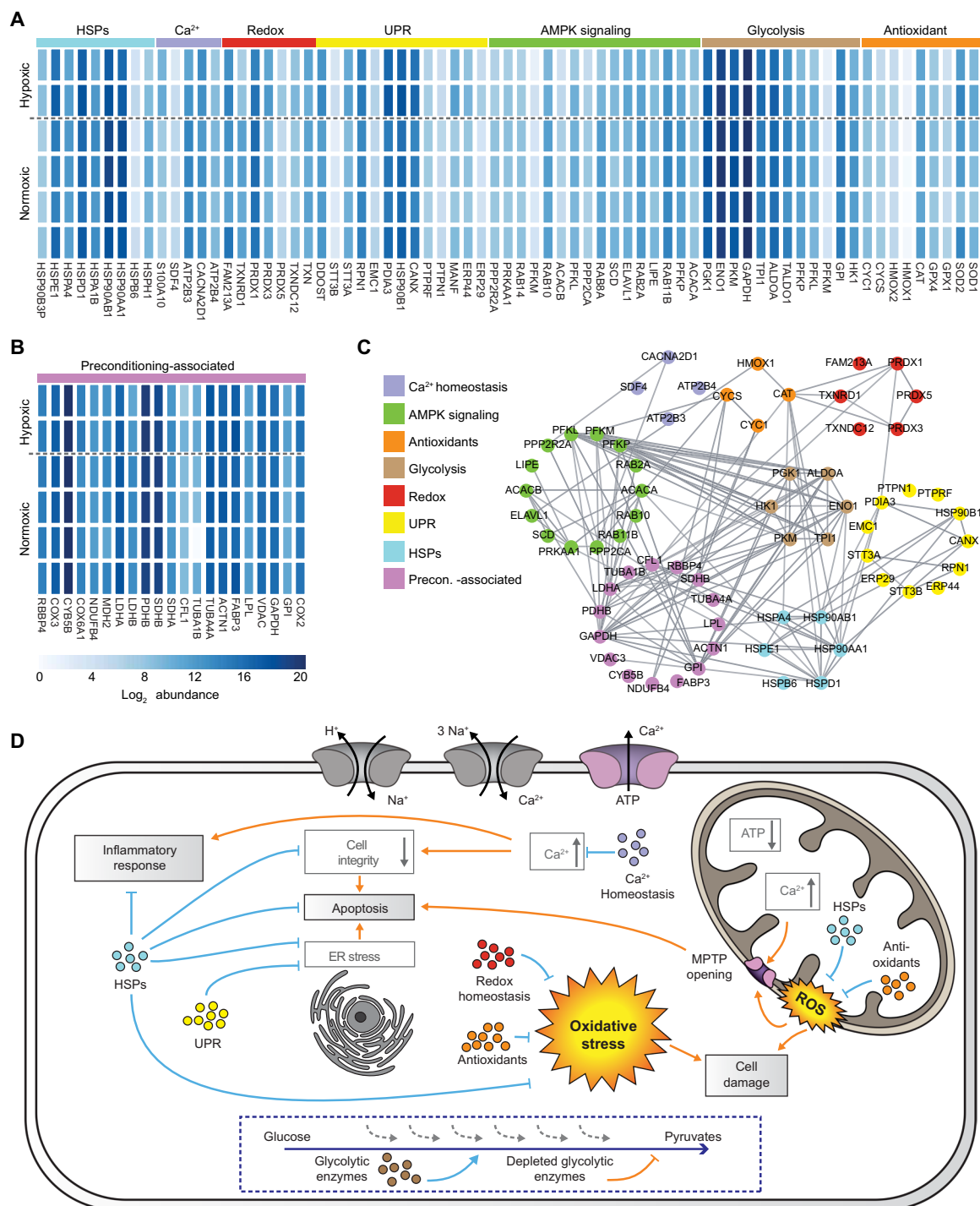
**Fig. 3. Endothelial EVs' effects on hCMs.** (A) Proteomap illustrating the KEGG orthology terms and relative differences in mass abundance for the significantly ( $P < 0.05$ ) differentially expressed proteins (DEPs) in EEV-treated ( $N = 3$  samples), compared with untreated ( $N = 2$  samples), hCMs. (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) 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 dataset. 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 in the DEPs. Each dot represents a single protein, whereas the clusters represent biological processes. The red color scale represents the individual  $P$  value for the enrichment of each protein in the dataset. The dashed horizontal line represents 0-fold change, whereas 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 biological process. (F) Oxygen consumption rate (OCR; mean  $\pm$  SEM) 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  $\pm$  SEM) was calculated as the difference between the maximum and baseline OCR. Significance relative to untreated control was tested with one-way analysis of variance (ANOVA). Statistical significance was presented by \* $P \leq 0.05$ , \*\* $P \leq 0.01$ .  $P$  values are detailed in data file S4.

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) 9 antioxidants, (ii) 9 of the 10 enzymes participating in glycolysis, (iii) 16 AMPK (adenosine monophosphate-activated protein kinase)-related proteins, (iv) 13 proteins involved in the protective unfolded protein response (UPR) pathway, (v) 7 proteins involved in cellular redox homeostasis, (vi) 9 heat shock proteins (HSPs) that are known to affect multiple pathological processes and ameliorate myocardial IRI, and (vii) 5 proteins related to calcium homeostasis.

In addition to proteins that may target the pathophysiological mechanisms initiated by IRI, we identified a group of proteins in the EEVs that are known to be down-regulated in cardiomyocytes during IRI (Fig. 4B). These proteins are simultaneously known to be

positively affected by ischemic preconditioning treatment. Ischemic preconditioning was previously found to up-regulate 30 of 64 genes that are down-regulated after myocardial IRI (41). Of these, 21 respective proteins were found in the EEVs. The majority of these are involved in energy production and conversion, as well as metabolism. In addition, proteins respective to 6 of the 34 down-regulated genes that were not affected by preconditioning were found in EEVs. These findings suggest that EEVs may induce a cardioprotective effect partly via pathways similar to those associated with ischemic preconditioning.

Protein-protein interaction network analysis of the protein clusters (Fig. 4C) identified a high degree of connectivity between the preconditioning-associated proteins and the seven identified protein groups that may target IRI pathophysiological mechanisms.



**Fig. 4. Identification of cardioprotection-related proteins in normoxic and hypoxic EEVs. (A)** Heat map illustrating the log<sub>2</sub> 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)** Heat map illustrating the log<sub>2</sub> 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 found 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 (A). Orange arrows stand for pathological pathways, and blue arrows indicate the counteractions.

Particularly, these are proteins heavily involved in cellular metabolism and repair (i.e., “glycolysis,” “AMPK,” and “HSP” protein groups). The observed high connectivity implies synergistic activity of the different clusters in modifying the cellular response to IRI, whereby multiple pathways can be targeted by EEVs to induce a cardioprotective effect. Together, these EV-residing proteins may assist in preservation of cellular integrity, structure, and function, as well as inhibit the onset of apoptotic signaling initiated by pathological IRI (Fig. 4D).

### Effect of EEVs on cardiac tissue viability after simulated IRI

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

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

To elucidate the cellular processes involved in the EEV-mediated rescue of viability, we compared the protein profiles of IRI-challenged hCMs with and without EEV treatment. We found ~1350 proteins that were significantly ( $P \leq 0.05$ ) differentially expressed in the EEV-treated population (data file S3) and analyzed their cellular component and biological process classifications. The proteomap was similar to that derived from the EEV samples, with DEPs including metabolic proteins, and protein expression/protein folding factors (Fig. 6A). The DEPs’ cellular component (Fig. 6, B and C) and molecular function (Fig. 6D and fig. S6) classifications showed dominant mitochondrial and catalytic activity-related proteins. This implies differences in the metabolic landscape of the EEV-treated injured cells. The biological processes related to the DEPs include cellular response to stress, respiration, energy derivation, glucose, and fatty acid metabolism, as well as protein targeting and folding

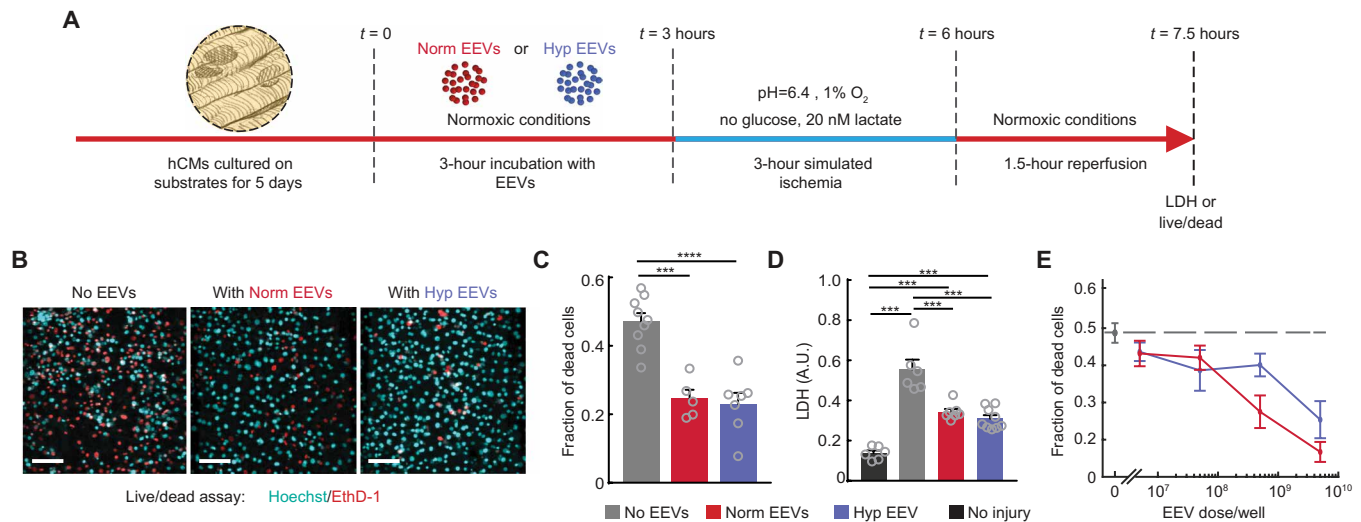
(Fig. 6E and fig. S7). Principal components analysis of injured/uninjured and EEV-treated/untreated hCMs demonstrated that EEVs only slightly affect the protein profile of uninjured cells, whereas the larger modifications induced by IRI were partially rectified by the EEV treatment (Fig. 6F).

The EEV-dependent effect on hCMs’ viability and protein expression after IRI may be mediated by cargo delivered into the cell via endocytosis or activation of a ligand-receptor response on the cell membrane without EEV internalization (24). To determine whether endothelial cell-derived EVs are internalized by cardiomyocytes, we produced fluorescent EEVs by labeling HUVECs with DiO, a green lipophilic tracer that is transferred to the cell membrane (Fig. 6, G and H). Using time-lapse microscopy, we observed internalization of these labeled EEVs by hCMs within 45 min (Fig. 6H, fig. S8, and movies S1 to S3). Therefore, we hypothesized that the cardioprotective effect of the EEVs was mediated by post-internalization cargo release. To test 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 inward 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). Lipid rafts are cholesterol-rich plasma membrane subdomains that form an inward budding vesicle. Filipin prevents shuttling into the cell by cholesterol sequestration that 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 IRI

Recovery of viable tissue contractile function after IRI is a key factor in determining the outcomes of MI 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 hICTs 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, 47). 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 after simulated IRI. Conditioning of hICTs with normoxic or hypoxic





**Fig. 5. EEVs are protective in myocardial ischemia-reperfusion injury.** (A) 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) and treated with normoxic (Norm, middle) and hypoxic (Hyp, right) EEVs (nuclei of live cells: cyan; dead cells: red; scale bars, 100  $\mu$ m). (C) Fraction of dead cells after reperfusion was quantified from the live/dead assay images. More than five fields of views for each sample were imaged with  $N = 9$  for control,  $N = 5$  for normoxic, and  $N = 7$  for hypoxic EEV treatment, tested with one-way ANOVA. (D) Injury after reperfusion was assessed by LDH fluorescence biochemical assay. LDH fluorescence readout was compared between an uninjured group to the control and EEV-treated groups after IRI, with  $N = 6$  for uninjured,  $N = 6$  for control,  $N = 7$  for normoxic, and  $N = 9$  for hypoxic EEV treatment. Data are represented as mean  $\pm$  SEM, in arbitrary units (A.U.), and tested with one-way ANOVA. Statistical significance was presented by  $^{**}P \leq 0.01$  and  $^{***}P \leq 0.001$ .  $P$  values are detailed in data file S4. (E) Fraction of dead cells after IRI as a function of EEV concentration ( $N = 3$  for each group).

EEVs had no direct effect on baseline contractile activity (Fig. 7, C and 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 after reperfusion (Fig. 7E, top). However, EEV-treated tissues continued contracting during simulated ischemia and displayed significant ( $P = 0.0005$  and  $P = 0.0172$  for normoxic and hypoxic EEVs, respectively) recovery of twitch stress after reperfusion (Fig. 7E). Whereas 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 myocyte (NRVM) tissues in the same HOC system (fig. S10A) and exposed them to the same simulated IRI protocol and EEV treatment. EEVs were internalized in the NRVMs (fig. S10B), yet their protective effect on NRVM-derived tissue viability and contractility was notably smaller (fig. S10, C to E).

## DISCUSSION

The key finding of this study is that vascular endothelial cell-derived EVs exert a protective effect against myocardial IRI in human tissue. 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.

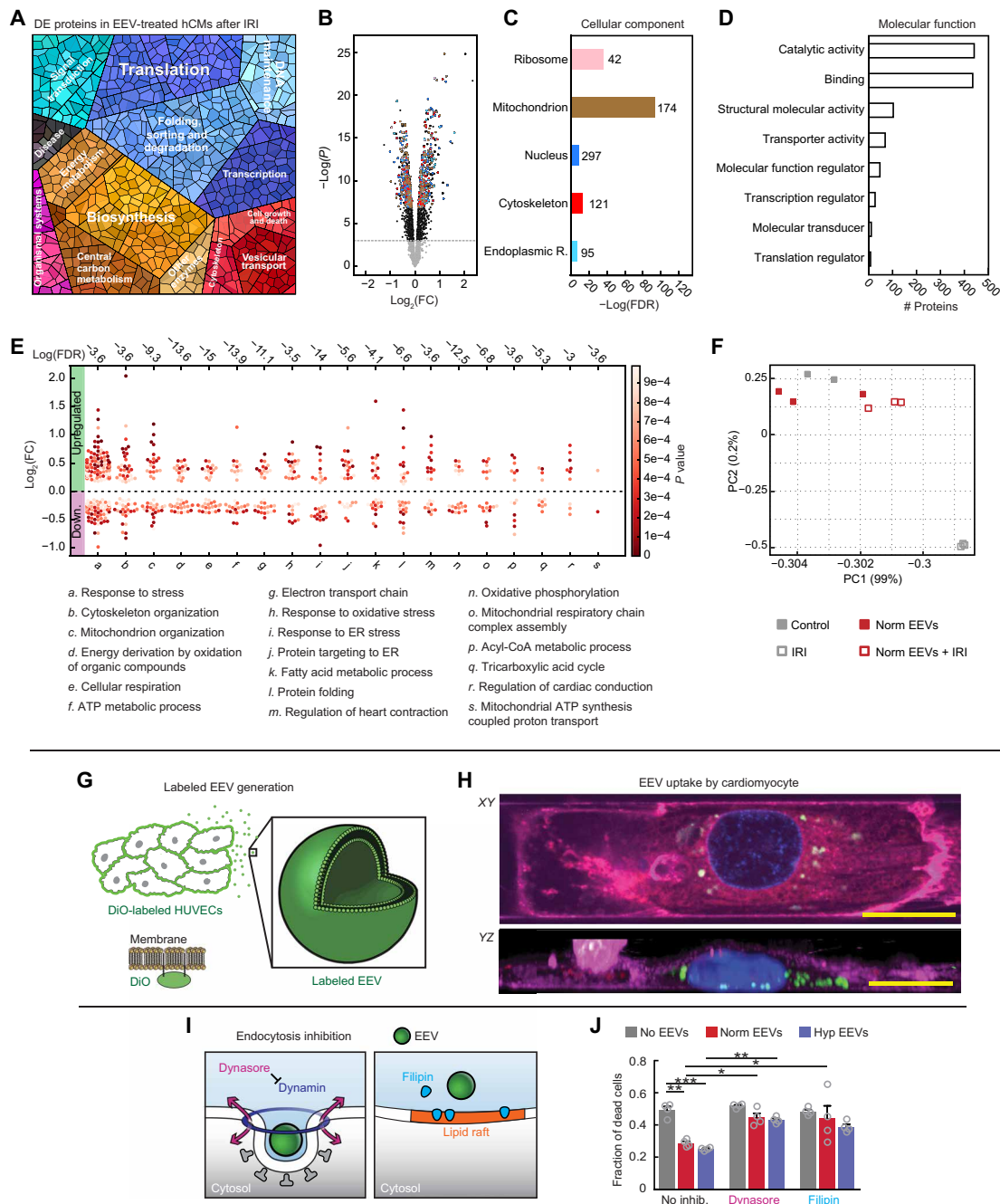
In control tissues without EEV treatment, the loss of contractile function was greater than the loss in viability. This mirrors in vivo IRI, where tissue contractile function is impaired due to not only cardiomyocyte death but also ATP depletion, abnormal calcium concentrations, and damage to contractile 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. Proteomic analysis revealed that EEVs shifted the protein profiles of IRI-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 antiapoptotic and proangiogenic 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 MI. Notably, they reported that the effect involved neovascularization and expansion of CPCs 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



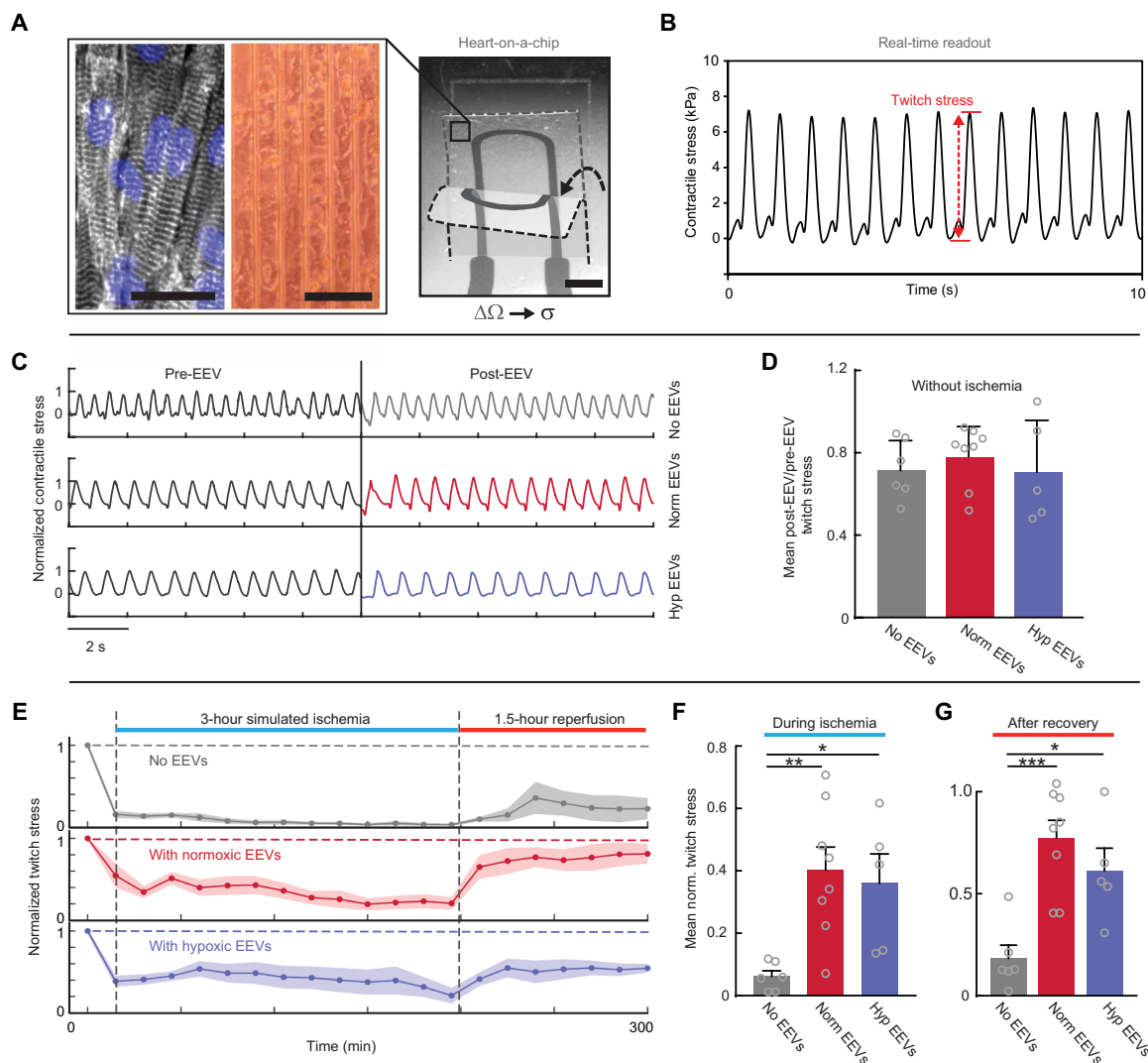
**Fig. 6. Endothelial EVs' effects on IR-injured hCMs.**

(A) Proteomap illustrating the KEGG orthology terms and relative differences in mass abundance for the significantly ( $P < 0.05$ ) 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 (C). (C) Cellular localization in the protein expression dataset. 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 dataset shows modification of catalytic activity and binding. (E) Dominant biological processes in the DEPs. Each dot represents a single protein, whereas the clusters represent biological processes. The red color scale represents the individual  $P$  value for each protein in the dataset. The dashed horizontal line represents 0-fold change, whereas all the dots above the line represent over-expressed proteins. The numbers above each cluster represent the false discovery rate, namely, the confidence level in the representation of the specific biological process. (F) Two-dimensional principal components 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 previous labeling of cultured HUVECs' plasma membranes. DiO-labeled EEVs could be detected using confocal microscopy. (H) XY and reconstructed YZ planes are presented 45 min after adding EEVs, showing internalization of the labeled EEVs (scale bars, 20  $\mu\text{m}$ ). 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 after IRI in the different treatment groups (nine experiment groups,  $N = 4$  for each group). Data are represented as mean  $\pm$  SEM and tested with two-way ANOVA. Statistical significance was presented by  $*P \leq 0.05$ ,  $**P \leq 0.01$ ,  $***P \leq 0.001$ , and  $****P \leq 0.0001$ .  $P$  values are detailed in data file S4.



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 does not require stem cells or stem cell 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



**Fig. 7. HUVEC-derived EVs rescue contractile function during IRI.** (A) Representative image of hCM-derived tissue in the HOC system. Left image shows immunostain for nuclei (blue) and sarcomeric  $\alpha$ -actinin (gray). Middle image shows an optical microscopy tissue culture image (scale bars, 100  $\mu$ m). On the right, a single PDMS cantilever with an embedded strain gauge is depicted (scale bar, 2 mm). As the hCT contracts, the cantilever bends, stretching the strain gauge and leading to increased resistance. Two different bending positions are overlaid to demonstrate the cantilever's motion. The relative resistance changes ( $\Delta\Omega$ ) are proportional to the generated twitch stress ( $\sigma$ ). (B) Example trace showing measured twitch stress. (C) Example traces of contractile stress before and after 3 hours of EEV incubation in untreated, normoxic, and hypoxic EEV-treated tissues. The contractile stress was normalized to average amplitude before treatment. (D) Mean ratio between average stress before treatment to average stress after a 3-hour EEV incubation period in control ( $N = 6$ ), normoxic ( $N = 8$ ), and hypoxic ( $N = 5$ ) EEV-treated tissues. No significant differences were found (one-way ANOVA). (E) Twitch stress amplitude normalized to baseline values throughout the simulated IRI. The bold traces present the average values over all the tested samples, and shaded areas represent the SEM. (F) Average twitch stress during ischemia relative to baseline. (G) Average twitch stress recovery relative to baseline for the three experimental groups. Error bars represent SEM.  $N = 6$  for control,  $N = 8$  for normoxic, and  $N = 5$  for hypoxic EEV-treated groups. Tested with one-way ANOVA. There was no significant difference between the two groups treated with EEVs. Statistical significance was presented by  $*P \leq 0.05$ ,  $**P \leq 0.01$ , and  $***P \leq 0.001$ . P values are detailed in data file S4.

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. Because the EEVs contain diverse protein and nucleic acid cargo, it is impossible to delineate, at this point, the differential effects of the various proteins or RNA cargo. However, a recent study by Pérez-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 observed here. Moreover, we observed a range of effects beyond increased survival, including improved function during and after ischemia, increased

cellular respiratory reserve, and slightly decreased ROS production in menadione-challenged cells. This implies that multiple factors likely contribute to the net effect.

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

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

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

In this study, we used a recently published human “Heart-on-a-Chip” *in vitro* IRI assay (28, 29) to examine the effects of endothelial

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

Among current interventions to reduce ischemic damage, ischemic preconditioning is the most efficient intervention in reducing infarct size and is known to improve cell survival and recovery of mechanical function by activating multiple pathways (69, 70). Preconditioning has been shown to decrease the fraction of dead cells by up to 50% and improve recovery of contractile force twofold, after only 90 min of ischemia and reperfusion in human *in vitro/ex vivo* models (69, 70). In comparison, we found that the fraction of dead cells was similarly reduced by 50% and that recovery of contraction was about four times higher in EEV-treated tissues than in control tissues after 3 hours of simulated ischemia and 90-min reperfusion. In addition, preconditioning hICTs with 10-min ischemia and 10-min reperfusion, followed by prolonged (3 hours) ischemia and reperfusion (90 min), resulted in lesser viability compared to EEV-treated, injured hICTs. This comparison suggests that the EEV-induced cardioprotective effect could be at least as effective as the protective effect induced by preconditioning.

Our findings indicate that EEVs could constitute a multitarget therapy for IRI, but several challenges are apparent for translating EVs into a clinically relevant therapy. For instance, the cell source used here for EEV derivation was venous endothelial cells. Although they produce an apparent cardioprotective effect, other cell types, for example, arterial endothelial cells, may be more relevant as a cell source for EVs. An adaptation of our current experimental design, where an assessment of the therapeutic effect of EEVs when administered before or immediately after reperfusion, is an important experimental milestone en route to future clinical deployment of EEVs. Similarly, studying the most effective time window for a cardioprotective effect will be necessary for translation of the findings presented here. A question remains regarding the duration of EEV cargo-induced effects, and how they vary with time after treatment. As a further obstacle for clinical translation, traditional cell culture may not be sufficiently scalable for high-throughput production of EVs. Last, issues such as dosing efficiency, clearance, targeted delivery,

and EV cargo optimization to increase therapeutic efficiency must still be evaluated. To optimize the treatment, an appropriate in vivo model should be designed, which could be facilitated by the HOC system as demonstrated here with both human- and NRVM-derived tissues. In that sense, the present results may represent a starting point for 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, identify lead cardioactive proteins, and assess the effect of EEVs on hICTs exposed to IRI. On the basis of analysis of EEV protein contents, we hypothesized that these EEVs are cardioprotective against IRI. We designed an hHOC system to test the effects of EEVs on hICT contractile function and used hCM 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 proteomic data, EEV proteomics were derived from four samples of normoxic EEVs and two samples of hypoxic EEVs. Proteomic analyses of cardiomyocytes were derived from three samples for each treatment group, except for the untreated, uninjured control group (two samples).

### EV isolation

EVs were isolated by ultracentrifugation, as previously published (71). Briefly, 12 hours after passage 5 or 6, HUVECs were washed once with warm phosphate-buffered saline (PBS) and the standard endothelial growth medium (EGM) was replaced with EGM containing 2% exosome-depleted fetal bovine serum (Invitrogen, Carlsbad, CA, USA). After 48 hours, culture medium was collected for “normoxic EV” isolation. Fresh exosome-free medium was then added to the flasks, and cells were then subjected to 3 hours of hypoxia (1% O<sub>2</sub>). Then, culture medium was collected for “hypoxic EV” isolation. The culture medium was divided into 50-ml conical tubes and centrifuged at 300g, 4°C, for 10 min. The supernatant was collected and centrifuged at 2000g, 4°C, for 20 min. Supernatant was collected and centrifuged at 10,000g, 4°C, for 30 min. Last, the supernatant was placed in thin-wall, Ultra-Clear, 38.5-ml tubes and centrifuged at 100,000g, 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 resuspended in sterile PBS. All the normoxic EV suspensions were pooled in a single tube, and the hypoxic EV suspensions were pooled in another. Then, a second ultracentrifugation

cycle was carried out under the same conditions. The EV pellet was then resuspended in 500 µl of PBS, or in 150 µl of lysis buffer (Covaris, Protein extraction buffer TP), if the samples were designated for protein 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.

### EV labeling

EV-producing endothelial cells were initially labeled using a lipophilic tracer before the last passage before being placed in the exosome-free, serum-enriched medium. Staining medium was prepared by adding 5 µl of 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 hours. Culture media were collected for EEV isolation after 48 hours, as described above. After EEV isolation, 20 µl of labeled and nonlabeled EEV suspensions was added to a black 384-well plate and loaded into a plate reader for fluorescence intensity measurement.

### Simulated IRI

To simulate IRI, cardiomyocytes were exposed to 3-hour ischemic conditions and 1.5 hours under reperfusion treatment. Ischemia was simulated by switching the complete culture medium to a Tyrode's-based ischemia buffer with 4 mM KCl, 115 mM NaCl, 1.8 mM CaCl<sub>2</sub>, 5 mM Hepes, 1 mM MgCl<sub>2</sub>, 0.33 mM NaH<sub>2</sub>PO<sub>4</sub>, 20 mM sodium lactate, with no glucose, and pH 6.4. O<sub>2</sub> was set to 1%. For reperfusion, O<sub>2</sub> was increased back to 21% and medium was changed back to complete culture medium (pH 7.4). To test EEVs for a possible cardioprotective effect, cardiomyocytes were incubated with 10<sup>9</sup> EEVs per well (“normoxic” or “hypoxic”), 3 hours before 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% O<sub>2</sub> for 1.5 hours. Last, 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-min ischemia and 10-min reperfusion before the prolonged ischemia. hCMs were preplated, seeded in well plates, and cultured for 6 days, as described above. On day 6, the cells were washed three 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 min. After 10 min 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 min, after which the prolonged ischemia



and reperfusion protocol was implemented. A live/dead assay was performed right after the 1.5-hour reperfusion period. In the same experiment, we used a second well plate with hCMs that were not preconditioned as a control.

### HOC real-time contractility assays

The HOC systems, multiwell devices with instrumented muscular thin-film cantilever substrates, were fabricated as previously described (29). Briefly, multilayered polydimethylsiloxane (PDMS) films (21 to 23  $\mu\text{m}$ ) 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  $\mu\text{m}$  tall and 4  $\mu\text{m}$  wide, spaced by 30- $\mu\text{m}$  grooves) guide the formation of anisotropic cardiac thin-film tissues. Cantilevers sized 3.2 mm by 4.2 mm were defined using a CO<sub>2</sub> laser (Epilog). A poly(*N*-isopropylacrylamide) film ensured that cantilevers could detach from glass substrates when cooled briefly to room temperature. After ultraviolet ozone (UVO) treatment and fibronectin incubation, hCMs were seeded on hHOC devices as described above and cultured for 6 days before IRI experiments. Real-time contractility data were recorded using a multichannel digital multimeter system (Keithley 3706a) and analyzed using a custom MATLAB (MathWorks Inc.) code as previously described (29).

### EV 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. After staining, the coverslip was transferred to an imaging chamber filled with 1 ml of 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 hours, using a 60 $\times$  objective (Olympus). Immediately after the first time point at which images were acquired, 10<sup>9</sup> 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  $\mu\text{M}$  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 filipin (1  $\mu\text{g}/\text{ml}$ ) (Sigma-Aldrich), 30 min before addition of EEVs (73). To test the effects of EEV uptake inhibition on the EEV-mediated cardioprotection, IRI was simulated after a 3-hour incubation with normoxic and hypoxic EEVs under the inhibited endocytosis and control conditions. After IRI, tissue viability was quantified by performing the viability assay described above.

### Preparation of hCMs for proteomic analysis

Human stem cell-derived cardiomyocytes were thawed, preplated, cultured, and dissociated as described above. Five million hCMs were reseeded in eight wells in a density of 10<sup>5</sup> cells/cm<sup>2</sup> and cultured for 6 days. On day 6, the media were changed for all the wells. Half of the wells were treated with 3  $\times$  10<sup>10</sup> EEVs (5  $\times$  10<sup>9</sup> EEVs per

10<sup>5</sup> cells, as used for the rest of the experiments). The other half were treated with vehicle. After 3 hours, two of the EEV-treated and two vehicle-treated wells were exposed to IRI, as described above. This procedure resulted in four experimental groups: (i) hCMs, (ii) hCMs + EEVs, (iii) hCMs + IRI, (iv) 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, and pellets were washed with PBS and then centrifuged again. This was repeated for three times overall. After the final centrifugation step, the supernatant was removed and the pellets were transferred to -80°C.

### Samplewise Pearson correlation analysis

Pearson product-moment correlation (74) 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  $\pm$  SEM 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 analysis of variance (ANOVA) followed by 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

[stm.sciencemag.org/cgi/content/full/12/565/eaax8005/DC1](http://stm.sciencemag.org/cgi/content/full/12/565/eaax8005/DC1)

Materials and Methods

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

Fig. S2. Molecular function GO classification of DEPs in hCMs treated with EEVs compared to untreated controls.

Fig. S3. Biological process GO classification of DEPs in hCMs treated with EEVs compared to untreated controls.

Fig. S4. ROS measurement in menadione-challenged human cardiomyocytes.

Fig. S5. Preconditioning versus EEV treatment effect on viability after IRI.

Fig. S6. Molecular function GO classification of DEPs in injured hCMs pretreated with EEVs compared with injured, untreated hCMs.

Fig. S7. Biological process GO classification of DEPs in injured hCMs pretreated 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. Nonsignificant cardioprotective effect of human EEVs on neonate rat ventricular myocyte-based heart-on-a-chip assay.

Table S1. Common contaminant proteins detected in EEV samples.

Table S2. GO terms and enrichment  $P$  values for overrepresented protein groups presented in Fig. 2 (C and 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.

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

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

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

Data file S1. Total EEV proteome raw data.

Data file S2. DEPs in EEV-treated versus untreated hCMs.

Data file S3. DEPs in IR-injured, EEV-treated versus injured, untreated hCMs.

Data file S4. Summary of statistical analyses and  $P$  values.

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[View/request a protocol for this paper from Bio-protocol.](#)

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study are present in the paper or the Supplementary Materials. All proteomic raw data are deposited at <https://massive.ucsd.edu/ProteoSAFe/private-dataset.jsp?task=3421c03b031d4315a46dc8c1875ef63d>. Scripts for proteomic data analysis are available at <https://github.com/spsheehy/Proteomicsv> and [https://github.com/harvardinformatics/YadidMetal\\_May\\_2020](https://github.com/harvardinformatics/YadidMetal_May_2020). Scripts for contractility data analysis were previously published (26).

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

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### Valuable vesicles

Extracellular vesicles, small membrane-bound particles released from cells, have been shown to have cardioprotective effects. Here, Yadid *et al.* analyzed the proteins contained in vesicles released from endothelial cells under normoxia and hypoxia and investigated cardioprotective effects on cardiac tissues in vitro. Using a human heart-on-chip composed of cardiomyocytes, the authors showed that endothelial cell-derived vesicles supported metabolic function, tissue contraction, and viability during ischemia-reperfusion injury. This study helps to elucidate the mechanism by which vesicles are cardioprotective in human tissue.

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