Proteome characterisation of extracellular vesicles isolated from heart

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Abstract

Intercellular communication within the heart is critical for cardiac function and often dysregulated in diverse cardiovascular diseases. While extracellular vesicles (EVs) are emerging as important mediators of cardiac signalling, their isolation remains a technical challenge which has impeded our understanding of cardiac EV (cEV) protein composition. Here, we utilised Langendorff-collagenase-based enzymatic perfusion coupled with differential centrifugation to isolate cEVs from mouse heart (yield 3-6 μg/heart). cEVs were ~200 nm in size, expressed markers of classical EVs (Cd63/81/9^{+}, Tsg101^{+}, Pdcd6ip/Alix^{+}), and were depleted in blood components (Alb/Fga/Hba). Mass spectrometry-based proteomic profiling revealed 1,721 proteins in cEVs, implicated in proteasomal and autophagic proteostasis, glycolysis and fatty acid metabolism; essential functions often disrupted in cardiac pathologies. There was striking enrichment of 942 proteins in cEVs compared to mouse heart tissue, which were implicated in EV biogenesis, antioxidant activity, and lipid transport, suggesting active cargo selection and specialised function. Interestingly, cEVs contain marker proteins for cardiomyocytes (Actn1/Myh6), cardiac progenitor cells (Cd34/Abcg2), B cells (Cd1d1/74), T cells (Cd55/14/38), macrophages (Cd163/36), smooth muscle cells (Cald1/Tagln), endothelial cells (Vwf), and cardiac fibroblasts (Ckap4), suggesting diverse cellular origin. Our data provide insight into potential cEV functions and enables future studies seeking to explore their role in cardiovascular diseases.

Statement of significance

Our understanding of the role of extracellular vesicles (EVs) in cardiac physiology and pathology is severely impeded by technical challenges in isolating cardiac EVs (cEVs). Here, we report an efficient method to isolate EVs from heart using collagenase-based perfusion that avoids mechanical disruption. This enabled us to gain a comprehensive insight into cEV proteome and their potential role in metabolism, proteostasis, and antioxidant activity – processes critical to heart function. This knowledge will give us valuable insights into signalling perturbations in pathological contexts and identify molecular leads for pharmacological intervention and design of therapies for managing cardiovascular pathologies.

1. Introduction

Intercellular communication between different cells of the heart is highly complex, tightly regulated, and plays critical physiological and pathological roles. This signalling is mediated through direct cell-cell, or cell-extracellular matrix (ECM) interactions, release of soluble secreted factors, and more recently extracellular vesicles (EVs).[1, 2] Dynamic interplay of these modes of intercellular communication collectively coordinate processes such as contractility, vasodilation, extracellular matrix (ECM) organisation, metabolism, etc., essential to normal cardiac function.[1-8] Therefore, understanding players of intercellular communication and how they potentially regulate the functions of cardiac cells will provide valuable insights into signalling perturbations in cardiac pathologies.

EVs, membranous vesicles released by cells into the interstitial space and circulation, are important mediators of intercellular communication.[9, 10] They can functionally transfer proteins, nucleic acids, and metabolites between cells in different physiological (stem cell differentiation,[11], innate immunity,[12] and reproductive biology[13]) and pathological progression (cancer,[14-17] autoimmune disease,[18-20] and neurodegeneration[21-23]) processes. Importantly, cardiac cells have also been shown to release EVs into the extracellular space [24] where they can be internalised by neighbouring cardiac fibroblasts and disseminate systemically.[25] These observations have engendered vested interest in potential function of cardiac EVs with several studies reporting function of EVs derived from cardiac cells *in vitro* in immune regulation,[26] glucose uptake,[27] angiogenesis,[28] fibroblast activity,[29, 30] and cardiomyocyte behaviour.[31-34] However, our understanding of EVs in the heart tissue remains limited, mainly due to technical challenges associated with isolation. EVs have been isolated from the heart using two main approaches, namely, mincing of fresh or frozen samples,[32, 35-

37] and enzymatic tissue digestion followed by mechanical dissociation and pipetting.[38] Such mechanical disruption invariably leads to cell rupture, thereby contaminating EV preparations with intracellular components and membranous debris, confounding their functional study and omics-based characterisation.

Here, we present a workflow for isolation of cardiac EVs (~200 nm, Cd63/81/9^{+}, Pdcd6ip/Alix^{+}, Tsg101^{+}) from mouse heart via Langendorff-based collagenase perfusion. Mass spectrometry-based proteomic analysis identified key processes that are enriched within cardiac EVs and their potential role in regulating cardiac function.

2. Experimental section

2.1 Animals

All animal care and experimentation were conducted in accordance with protocols approved by the Alfred Research Alliance Animal Ethics Committee.

2.2 Collection of EV containing perfusates

Hearts were excised from C57BL/6 and FVB/N animals who had been pre-treated with heparin (1 unit/g body weight) 30 min prior to euthanising with Lethabarb (300mg/kg) and cervical dislocation. Hearts were cannulated through the aorta using a 22G cannula, stopping before the aortic valve (fixed with thread), and attached to a Langendorff apparatus within 5 min of death to allow for perfusion through the coronary arteries. The aorta underwent retrograde perfusion (wash) for 5 min with oxygenated, 37°C perfusion medium (5.4 mM KCl, 3.5 mM $MgSO_4$, 0.05 mM sodium pyruvate, 20 mM $NaHCO_3$, 11 mM glucose, 20mM HEPES, 23.5 mM sodium glutamate, 4.87 mM sodium acetate, 56 µM phenol red, 10 mM 2,3-butanedione monoxime, 5 mM creatine, 30 mM taurine, 0.1 IU/mL insulin, gassed with 95% O_2 and 5% CO_2, pH 7.25) at flow rate 2-3 mL/min with medium passing through a 0.45 µm filter before entering the heart, the wash perfusate was collected as it left the heart. After 5 min, medium was replaced with 10 mL digestion medium (perfusion medium, 3 mg/mL collagenase II (ScimaR batch #40A19951, 0.2% BSA) and the heart was placed in the heated reservoir with continuing perfusion. After 20-25 min the heart was removed and digestion perfusate passed through a 0.45 µm filter before collection.

2.3 Extracellular vesicle isolation

The perfusates from the wash and digest were collected and immediately centrifuged at 500 x *g* and 2,000 x *g* (4°C) to remove any cellular debris. Supernatant underwent ultracentrifugation at 100,000 x *g* (1 h, 4°C) to pellet EVs from perfusate (EVs from digest (cEV) and wash (wEV). Pellets were washed twice by resuspension in PBS and centrifugation at 100,000 x *g* (1 h, 4°C), final pellets were resuspended in 30 µl and stored at -80°C.

2.4 Soluble factor concentration

The supernatant containing soluble factors (SF, SF from digest (dSF) and wash (wSF)) following ultracentrifugation was concentrated using Amicon® Ultra-3 Centrifugal Filter Unit (UFC800324, 3kDa cut-off) as per manufacturer's instructions (4°C, 4,000 *g*) and stored at -80°C.

2.5 Nanoparticle tracking analysis

Particle size distribution of EVs isolated from perfusates (cEV and wEV) were determined by nanoparticle tracking analysis (NanoSight NS300, Malvern). cEV and wEV were captured and analysed using camera level = 10, screen gain = 10, detection threshold = 10, flow rate = 100, and temperature = 25°C. Data obtained were analysed through NTA software (Version 3.2.16**,** ATA Scientific).

2.6 Protein quantification

Protein quantification was performed using the microBCA™ Protein Assay Kit (Thermo Scientific, 23235) as per manufacturer's instructions.

2.7 Proteomics: extracellular vesicles and soluble factors

cEV/wEV (n=3) and dSF/wSF (n=4) were solubilised in 2% (v/v) sodium dodecyl sulphate (SDS), 50 mM triethylammonium bicarbonate (TEAB), pH 8.0, centrifuged at 16,000g for 10 min at 4°C, and quantified by microBCA (Thermo Fisher Scientific). For mass spectrometry-based proteomics, samples (EV = 3-5 μg, SF = 10 μg) were normalized and reduced with 10 mM dithiothreitol (DTT) for 45 min at 25°C followed by alkylation with 20 mM iodoacetamide for 30 min at 25°C in the dark. The reaction was quenched to a final concentration of 20 mM DTT. Magnetic beads were prepared by mixing Sera-Mag Speed Beads A and B at 1:1 (v:v) ratio and washing twice with 200 µL MS-water. Magnetic beads were reconstituted to a final concentration of 100 µg/µL. Magnetic beads were added to the samples at 10:1 beads-to-protein ratio and ethanol added for a final concentration of 50% (v/v). Protein-bound magnetics beads were washed three times with 200 µL of 80% ethanol and reconstituted in 50 mM TEAB and digested with trypsin (Promega, V5111) at a 1:50 enzyme-to-substrate ratio for 16 h at 37 °C at 1000 rpm. The peptide mixture was acidified to a final concentration of 2% formic acid, 0.1% trifluoroacetic acid (TFA) and centrifuged at 20,000*g* for 1 min. The peptide digests were kept frozen at -80°C and dried by vacuum centrifugation, reconstituted in 0.07% trifluoroacetic acid, and quantified by Fluorometric Peptide Assay (Thermo Scientific, 23290) as per manufacturer's instructions.

2.8 Proteomics: heart tissue homogenisation

Frozen heart tissues (-80°C, single thaw) were homogenized in 8 M urea, 50 mM HEPES lysis buffer pH 8.0, with HALT protease and phosphatase inhibitor (Thermo Fisher Scientific, 78444) on ice by Tissue Ruptor (Qiagen) followed by tip-probe sonication (Misonix S-4000 Sonicator, 3 cycles of 10 s, amplitude 23). Tissue homogenates were centrifuged at 20,000 g for 30 min, and supernatants were collected and quantified by microBCA (Thermo Fisher Scientific).

2.9 High-pH fractionation of heart tissue proteome

For heart tissue proteome, peptides (generated using single-pot, solid-phase-enhanced sample separation (SP3), Section 2.7) were fractionated by high pH separation, with ~10 µg peptide lysate fractionated into 10 fractions using in-house prepared C18 StageTips (Empore). Peptides were reconstituted in 25 mM ammonium formate (AF) in MS-grade water, pH 10. StageTips were primed sequentially with 250 µL of 100% acetonitrile (ACN), 50% ACN in 25 mM AF, pH 10 and 25 mM AF in MS-grade water, pH 10 by centrifuging at 1500 g for 5 min. Peptides were eluted using 2-50% ACN in 25 mM AF, dried by vacuum centrifugation, and reconstituted in 0.07% TFA in MS water, quantified by Fluorometric Peptide Assay and normalized by volume to 3 µL (100-500 ng).

2.10 Proteomic liquid chromatography–tandem mass spectrometry

Peptides were analysed on a Dionex UltiMate NCS-3500RS nanoUHPLC coupled to a Q-Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer equipped with nanospray ion source in positive mode as described.[39, 40] Peptides were loaded (Acclaim PepMap100 C18 5 µm beads with 100 Å pore-size, Thermo Fisher Scientific) and separated (1.9-µm particle size C18, 0.075 × 250 mm, Nikkyo Technos Co. Ltd) with a gradient of 2–28% acetonitrile containing 0.1% formic acid over 110 mins at 300 nL min^{-1} at 55°C (butterfly portfolio heater, Phoenix S&T). An MS1 scan was acquired from 350–1,650 *m*/*z* (60,000 resolution, 3×10^6 automatic gain control (AGC), 128 msec injection time) followed by MS/MS data-dependent acquisition (top 30) with collision-induced dissociation and detection in the ion trap (15,000 resolution, 1×10^5 AGC, 60 msec injection time, 28% normalized collision energy, 1.3 *m*/*z* quadrupole isolation width). Unassigned precursor ions charge states and slightly charged species were rejected and peptide match disabled. Selected sequenced ions were dynamically

excluded for 30 sec. Technical replicates were performed for all analyses (n=2). Data was acquired using Xcalibur software v4.0 (Thermo Fisher Scientific). A list of samples and RAW data is available in ProteomeXchange. #PXD023570.

2.11 Database searching and protein identification

Identification and quantification of peptides was performed using MaxQuant (v1.6.14.0) [41] and Andromeda[42] as previously described.[43] Tandem mass spectra were searched as a single batch against *Mus musculus* (mouse) reference proteome (55,398, downloaded 15/01/2020) supplemented with common contaminants. Search parameters were as follows: carbamidomethylated cysteine as fixed modification, oxidation of methionine and N-terminal protein acetylation as variable modifications, trypsin/P as proteolytic enzyme with ≤ 2 missed cleavage sites, search tolerance 7 ppm, fragment ion mass tolerance 0.5 Da, <1% false discovery rate on peptide spectrum match with target-decoy approach at peptide and protein levels, match between runs selected, and label free quantification (LFQ) algorithm employed.

2.12 Data analysis and informatics

Protein lists for samples were generated in Perseus (Version 1.6.14.0)[44] based on ≥ 2 peptides and present in ≥ 60% sample replicates. Principal component analysis was performed in Perseus for proteins present in all replicates of all samples. Annotation of proteins into functional classes was performed using The Cell Surface Protein Atlas.[45] Protein lists were downloaded from Vesiclepedia[46] and Exocarta[47] for comparison. Venn diagram values were generated using the Bioinformatics Evolutionary Genomics web tool (http://bioinformatics.psb.ugent.be/webtools/Venn/). Column and bar graphs were generated using GraphPad Prism (Version 8.1.2). GO and functional enrichment annotations were retrieved by submitting protein accession IDs to DAVID Bioinformatics Resources

(https://david.ncifcrf.gov/).[48, 49] Hierarchical clustering was performed in Perseus using Euclidian distance and average linkage clustering, with missing values imputed from normal distribution (width 0.3, downshift 1.8). Pathway enrichment map analysis was performed using Cytoscape (Version 3.8.2, p-value ≤ 0.05, FDR ≤ 0.1 cut offs).

3. Results

3.1 EV isolation from cardiac tissue

EVs have previously been isolated from hearts and cardiac tissues using mechanical dissociation[32, 35, 36, 38] which invariably results in cell lysis and intracellular contamination of EV preparations. To avoid this, we used collagenase II to gently hydrolyse the ECM and loosen cardiac tissue structure, a procedure commonly used for the isolation of cardiomyocytes.[50-53] Freshly isolated intact mouse hearts were perfused with perfusion medium (wash step) to deplete abundant blood proteins, followed by collagenase II-perfusion medium (digest step) (**Fig 1A/B)**. This allowed us to obtain perfusates containing SFs and EVs present within the intercellular space. These perfusates were subjected to differential centrifugation to separate EVs from SFs (**Fig 1A**).

3.2 Proteomic profiling of cardiac EVs

To identify the EV-containing fraction, we performed proteomic profiling of EVs and SFs obtained from wash and digest heart perfusates (**Fig 2A, Supplementary Table S1**). Principal component analysis of proteins identified (and their abundance) revealed that fractions were distinct (**Fig 2B**). Classical EV markers[54] (Cd63, Pdcd6ip/Alix, Tsg101) were enriched in EV fraction obtained from digest perfusate (**Fig 2C**). Importantly, during collagenase II-based digestion, levels of cytoplasmic proteins (Actc1, Gapdh, Tuba1b) and clinical markers of

cardiac damage[55] (Ldhb, Mb, Tnnt2) did not increase (**Fig 2D/E)**, and we did not detect proteins activated in apoptotic processes (Casp3/9, Bax, Bak) (**Supplementary Table S2**). Additionally, levels of Alb, Fga, and Hba were highest in wash fractions, suggesting successful perfusion of heart and depletion of abundant blood components from EV preparations (**Fig 2F**).

Interestingly, of the 1,721 proteins identified in cEV, 1,575 were previously reported in EV databases (Exocarta[47] and Vesiclepedia[46]) (**Fig 2G, Supplementary Table S2**). Commonly identified proteins include those implicated in EV biogenesis: vesicle formation (Tsg101, Vps4b, Pdcd6ip/Alix, Cd9/63/82, Sdcbp), cargo sorting (Hnrnpa2b1, nSMase/Smpd2, Kras), membrane fusion and release (Rab11b/35/7, Vti1b).[56] On the other hand, 139 proteins in cEVs were not reported in EV databases, 12 of which are enriched in cardiac tissue (enzymes Dhrs7c, Hhatl, Hacd1, cardiac regulators Pln, Hrc, Csrp3, structural components Tnnt2, Sgcg, signalling factor C1qtnf9, and mitochondrial components Mpc2, Cox7a1, Ucp1) compared to other organs.[57] We also report 927/1,721 cEV proteins are co-identified in EVs from rat hearts isolated using mechanical extraction and purified using density gradient separation (including known cardiac components Casq2, Mybpc3, Ryr2, and Tnnt2), in addition to 794 proteins unique to this current study (including EV markers Cd63, Cd82, Chmp2/3/4/5, Vamp3/5/8) (**Supplementary Table S2, Supplementary Fig 3**).[37]

Furthermore, nanoparticle tracking analysis revealed that these EVs were ~100-300 nm (mean ~200 nm) in diameter (**Fig 2H**), consistent with previous reports for cardiac cell-derived EVs.[26, 30, 32] Typically, we obtained 3-6 µg of EVs (~2.5-8 × 10^8 particles) per heart; we refer to these EVs as cardiac EVs (cEVs).

3.3 Functional annotation of global cardiac EV proteome

Next, we dissected the cEV proteome to gain insight into potential function. Annotating cEV proteome into functional protein classes[45] revealed 85 cluster of differentiation (Cd14/163/177//200), 7 tetraspanins (Cd63/81/82/9, Tspan8/9, Upk1b), 82 transporters (Vamp3/5/8, Stx2/4/7/8/12, Slc27a1/2/4, Atp2b1/4), 64 receptors (including 16 integrins, Epha4, Egfr, Npr1/2/3, Pdgfra/b), and 126 enzymes (including Adam10/17/9, Ndufa1/4, Cpt1a/b, Acaa2/t2) (**Supplementary Table S2**).

Gene Ontology (GO) analysis for cellular component revealed that cEVs were enriched in extracellular exosome (Cd63/82/9, Tsg101, Chmp2/3/4/5), membrane (Anxa1, Egfr, Pdgfra/b), and focal adhesion (Ctnna1, Tln2, Zyx) proteins (**Fig 3A, Supplementary Table S3**). Molecular function and biological processes enriched in cEVs included protein stabilisation (Cdc37, Clu, Bag3), poly(A) RNA binding (Ybx1, Eif3a/5a, Pabpc1), and cell-cell adhesion (Epcam, Bsg, Ctnna2) (**Fig 3A**).

Because EVs regulate several signalling pathways and are important in metabolic reprogramming,[58, 59] we performed KEGG pathway analysis on cEV proteome (**Supplementary Table S3**). This revealed enrichment of signalling pathways including cGMP-PKG (Kng-1, Npr1, Npr2), adrenergic signalling in cardiomyocytes (Prkaca, Mapk1/3, Gnas) and Rap1 (Rab1a/1b) (**Fig 3B**). We have manually curated key signalling pathways and proteins in cEVs that are known to function in heart physiology; several of which have been shown to be functionally delivered or influenced by EVs in various cells/tissues, including heart (**Table 1**).

Metabolic pathways enriched include proteasome (Psma1, Psmb1, Psme1), pentose phosphate (G6pd, Taldo1, Pgd), glutathione (Gpx1/3/4/7/8, Gstm1/2/5, Gsta3/4), and glycolysis (Hk2,

Gpi1, Pkm) (**Fig 3C, Table 1**). Strikingly, cEVs contain proteins for each step of glycolysis, these include rate limiting enzymes Pfkl/m/p and Hk2 (**Fig 3D**). Indeed, these signalling and metabolic pathways are essential for cardiac homeostasis[60-64] and can be regulated by EV-mediated intercellular communication in the heart (**Table 1**).

3.4 Comparison with heart reveals biological processes enriched in cEV

Although EV proteome represents a subset of parental tissue proteome, it is now well established that cells selectively sort proteins within the EVs to perform diverse functions.[65-67] We next compared cEV proteome with heart tissue proteome (3,244 proteins identified, **Supplementary Table 1)**. Indeed, cEV and heart tissue were distinct; cEVs were enriched in EV markers (Cd63/81/9, Flot1/2, Pdcd6ip), while heart tissue was enriched in intracellular organelle proteins (Uqcrh and Ndufs6 (mitochondrial), Lmna and H1-2 (nuclear), Rplp2 and Rpl32 (ribosomal)) and contractile apparatus (Tnnt2 and Tnni3) (**Fig 4A**). This highlights selective packaging of proteins in cEVs; specifically, 942 components were selectively packaged in cEVs (538 unique and 404 enriched (log2(fold change) ≥1 over heart tissue) (**Fig 4B**), and were enriched for GO terms (cellular component) including endosome (Cd63/81/82/9, Pdcd6ip/Alix, Chmp2/3/4/5), cell surface (Anxa1, Itga1/2, Itgb1/2, Bsg), and membrane raft (Raftn1, Flot2, Cav1/2, Gnai1/2/3) (**Figure 4C**). EnrichmentMap analysis of GO biological process revealed enrichment of adaptive immunity, cell surface interactions, lipid transport, and peptide processing (**Fig 4D/E, Table 1, Supplementary Tables S4&5**). Proteins enriched in cell surface interactions in cEVs include those implicated in heterotypic cell-cell adhesion (Ptprc, Itgb2, Itgav), cell adhesion (Ncam, Alcam, Bcam, Icam, Pecam), and integrin-mediated signalling pathway (Adam9/17, Itgam, Itgb3). Adaptive immunity components for antigen processing and presentation include major histocompatibility complex (MHC) class I/II

subunits (H2-aa/-ab/d1/k1/q10) used by antigen presenting cells (APCs, dendritic cells, macrophages, B cells) to regulate T cell response.[68] cEV enriched peptide processing-processes (proteolysis and regulation of blood pressure) contain enzymes (Ace, Anpep, Cma1, Enpep, Lnpep, Mme) for the processing of key signalling angiotensin peptides involved in cardiac physiology.[69] Additionally, cEVs were enriched in lipid metabolism proteins, including those involved in lipid transport (Cd36, Fabp3, Fatp1, Ldlr, Vldlr). Thus, our data show that cEVs contain selectively packaged proteins associated with blood pressure regulation, angiogenesis, immune response, and myocardial remodelling processes which are implicated in cardiac function.[68-73]

4. Discussion

EVs are potent and complex mediators of intercellular communication, transferring selectively packaged cargo between cells to induce functional response.[27, 74, 75] In the heart, recent evidence suggest that EVs regulate immune response,[26] fibroblast gene expression and activation,[29, 30] cardiomyocyte size,[31, 32] and vascularisation,[28] regulation of which is often disrupted in cardiac pathologies. However, our understanding of cardiac EV protein composition remains limited due to technical challenges associated with EV isolation from cardiac tissue. In this study, we isolated cEVs (~200 nm, Cd63/81/9^{+}, Pdcd6ip/Alix^{+}, Tsg101^{+}) from mouse heart via collagenase perfusion and provide a comprehensive proteomic profile of these EVs to reveal insights into their composition and putative function.

Previously, cardiac tissue-derived EV isolations have involved 'dicing' or 'mincing' of frozen tissue.[36, 37] However, tissue freezing and homogenisation results in tissue damage and cell disruption, thereby contaminating EV preparations with intracellular components and membrane debris. Another major challenge includes removal of blood components that would otherwise impede mass spectrometry-based characterisation of cEVs. In this study, we immediately perfused freshly isolated intact heart to deplete blood components (Alb, Fga, Hba), and followed with collagenase perfusion. Collagenase II is a protease which cleaves collagen (Pro-X-Gly-Pro), thereby disrupting the ECM and heart tissue structure, while maintaining cardiomyocyte viability.[50-53] Because cardiac cells release EVs into the intercellular space,[24] collagenase-based disruption of the ECM potentially facilitates release of EVs during perfusion. Indeed, the particle yield of cEVs isolated following digest was higher compared to that in the wash step (**Supp Fig. S2**). While we expect some level of cell damage using collagenase-based perfusion which could contribute to increased levels of EVs

observed in digest perfusate, we did not observe an increase in markers of cardiac damage or loss-of-cellular integrity/ intracellular components or proteins activated in apoptotic processes in cEVs (Actc1, Gapdh, Tnnt2, Mb, Casp3/9). Thus, we provide an efficient way to isolate EVs from heart that bypasses the need for mechanical disruption.

cEVs contain an array of fatty acid (FA) binding proteins (Fabp3/4/5) and FA transport proteins (Fatp1/2/4, Cd36) which have been implicated in cardiac FA uptake.[72, 73] Previous reports have identified Fabp4[76] and Fatps[77] in circulating EVs, and demonstrated that $Cd36^{+}$ EVs take up FA from circulation and deliver them to cardiomyocytes *in vitro* and *in vivo*,[78] whereby FAs are preferentially used to generate large volumes of ATP, required for cardiac contraction.[63, 79] Identification of these proteins in cEVs supports their role in cardiac substrate delivery. Moreover, cEVs contain enzymes required for glycolysis (Hk2, Gpi1, Pfkm, Aldoa, Tpi1, Gapdh, Pgk1, Pgam2, Eno1, Pkm), including rate limiting enzymes Pfkl/m/p and Hk2.[80] In addition to FA metabolism, the heart also performs glycolysis in the cytoplasm to produce ATP, NADH, and pyruvate.[63] ATP produced during cytoplasmic glycolysis is particularly important for regulation of ion pumps and channels in the cell periphery, contributing to the maintenance of essential ion gradients associated with contraction.[81] Importantly, EVs have been shown to regulate glucose metabolism between cells,[82, 83] with cardiomyocyte-derived EVs transferring glucose transporters between cells to increase glucose uptake.[83] Because EVs have been shown to contain functional glycolytic enzymes,[84] whether cEVs can regulate glycolysis between cells in the heart remains in question. Given altered substrate utilisation is associated with cardiac pathologies,[63, 64] altering composition of cEVs may provide an opportunity to restore balance through external metabolic regulation.

Another interesting finding was enrichment of proteasomal subunits (Psma1-7, Psmb1/4/5) and components involved in proteasomal regulation (Psmc1-5, Psmd1/2/3/6/7, Psme1/2) in cEVs. In the heart high levels of active protein breakdown can be mediated by proteosomes.[79] Failure of which are associated with accumulation of misfolded proteins, or proteotoxicity which can contribute to cardiac pathologies.[85, 86] Because proteasome units within mesenchymal stem cell (MSC)-derived EVs are capable of *de novo* proteasome activity,[87] whether cEVs can mediate functional transfer of active proteosome units to regulate proteostasis in the heart warrants investigation. Additionally, the proteasome system works in conjunction with chaperones and autophagy to regulate proteostasis in the heart.[86, 88] Indeed, cEVs contain molecular chaperones associated with protein stability (Hsp90aa1/b1, Hspa1a/12b/13, Hspb1, Dnaja1/c13). Furthermore, we identified activators of AMPK signalling (Adipoq, C1qtnf9/Ctrp9) and AMPK kinase subunits (Prkaa1, Prkag1), a pathway which acts as a key modulator of proteostasis in the heart, regulating levels of autophagy for cardioprotection under pressure overload induced cardiac stress.[89, 90] EVs isolated from induced pluripotent stem cell-derived cardiomyocytes are able to enhance autophagy in cardiomyocytes and improve cardiac function and viability after myocardial infarction.[91] Moreover, EV transfer of Hsp40 (Dnaj proteins) regulates proteostasis in recipient proteotoxic cells *in vitro* and *in vivo*.[92] Whether cEVs assist in cardiac proteostasis through delivery of heat shock proteins, proteasomes, and promotion of autophagy warrants further investigation.

We also observed a striking enrichment in cEVs in antioxidants, including Cat, Sod1/2/3, Gpx1/3/4/7/8, Gstm1/2/5, Gsta3/4. Tight regulation of redox signalling is central to cardiac physiology (ion channels, contraction) and pathology (fibrosis, hypertrophy), whereby excess oxidants, such as reactive oxygen species (ROS), result in oxidative stress, cell damage and cardiac pathologies.[93-95] While EVs have been shown to deliver functional antioxidant

enzymes between cells (fibroblasts and hepatic cells[96, 97]), and particularly in the heart to reduce oxidative stress during myocardial infarction,[98] whether they regulate antioxidant levels in cardiac physiology remains unknown. Of note, cEVs also contain pentose phosphate pathway (PPP) proteins (G6pd, Taldo1, Pgd, H6pd, Tkt, Gpi1) that regulate NADPH production required for antioxidant machinery;[64] recently these PPP proteins in EVs were shown to be functionally active.[99] Collectively, this suggests that cEVs have the potential to act as independent ROS scavengers or donate antioxidant machinery to recipient cells to assist in redox regulation.

Several outstanding questions remain, primarily which cells in the heart produce these cEVs and whether they are of non-cardiac origin. Comparison of cardiosphere cell-specific and immune cell markers listed in CellMarker[100] revealed cEV contained markers for cardiomyocytes (Actn1, Myh6), cardiac progenitor cells (Cd34, Abcg2), B cells (Cd1d1, Cd74), T cells (Cd55, Cd14, Cd38), macrophages (Cd163, Cd36), smooth muscle cells (Cald1, Tagln), endothelial cells (Vwf), and cardiac fibroblasts (Ckap4), suggesting the production of cEVs by a range of cardiac cells. This echoes the incredibly complex and interconnected nature of cardiac signalling.[1] To investigate potential non-cardiac tissue sources of EVs, we compared cEVs with the Human Protein Atlas.[57] This revealed 60 cEV proteins reported to be ‘not detected’ in heart, but ‘elevated’ in bone marrow (6), brain (4), adrenal glands (2), kidney (7), pancreas (4), intestine (14), liver (32), lymphoid tissues (3), and lung (2) (**Supplementary Table S2**). Additionally, cEVs contain integrin expression patterns previously associated with EV homing to lung (Itga6/b4, Itga6/b1) and liver (Itgav/b5),[101] potentially contributing to their dissemination to lung and other organs.[25, 102] With many diseases involving multiple organs (e.g. cardiorenal and metabolic syndrome[103, 104]), gaining further insight into the role of EVs and how they can be dysregulated in organ crosstalk is

essential. Developments in the study of organ profiling & mapping,[57] inter-organ communication,[105] and fluorescence-based *in vivo* tracking[106] will offer tools for future exploration.

In summary, we have shown the successful isolation of EVs from a mouse heart and performed proteomic profiling to explore their composition. We propose cEVs may act as cardiac regulators by influencing peptide signalling, antigen presentation, proteostasis, metabolism, and oxidative stress (**Fig 5**). Further investigations into cEV origins, both cell and organ, will provide insight into biodistribution and inter-organ communication to develop our understanding of systemic pathologies. Additionally, isolation of cEVs from models of cardiac pathologies will provide insights into disease mechanisms, allowing development of therapeutic strategies.

Supporting Information

Supporting information is available from the Wiley Online Library or from the author.

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Author Contributions

BC carried out the majority of experiments and data analysis. JL and AM performed cannulation and Langendorff perfusions. HF assisted with heart tissue sample preparation. BC, AR, and DWG wrote, reviewed, and edited the manuscript. JRM facilitated the Langendorff perfusion studies and reviewed and edited the manuscript. All authors approved the final manuscript.

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Data and Software Availability: The accession number for the mass spectrometry data reported in this paper is PeptideAtlas Consortium via the PeptideAtlas proteomics repository: PXD023570

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Figures and legends

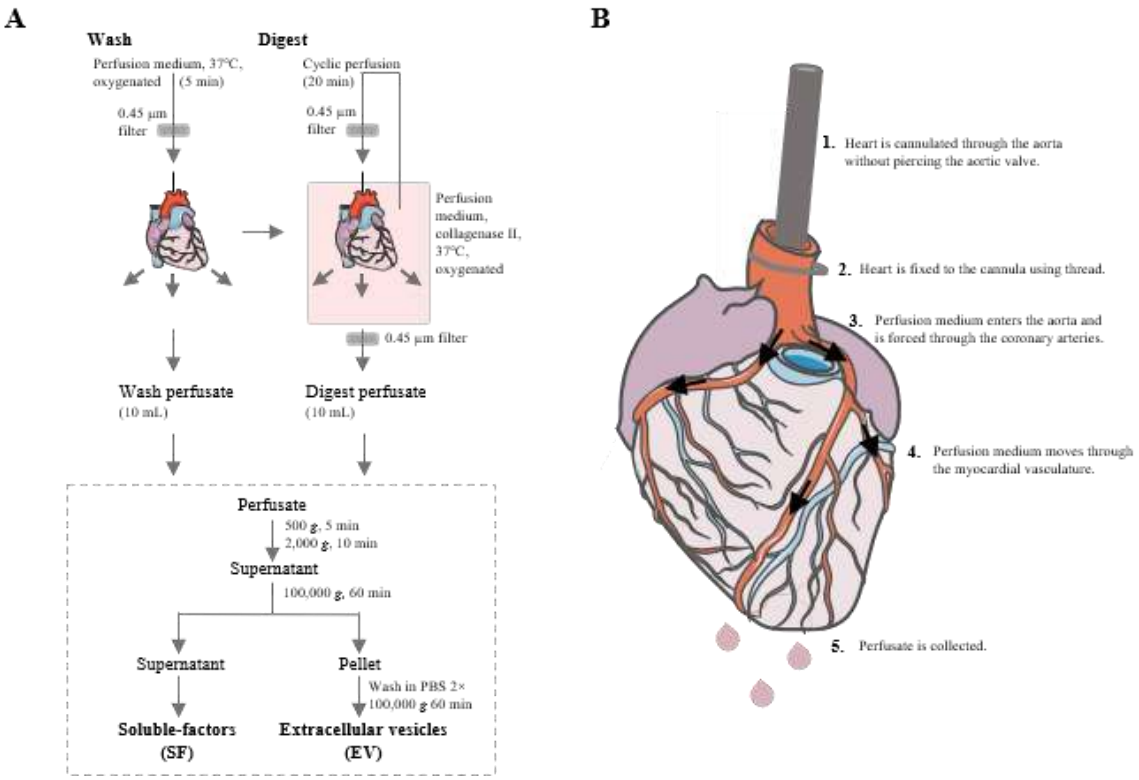

Figure 1. Isolation of extracellular vesicles from mouse heart. (A) Workflow for isolation of extracellular vesicles and soluble factors from mouse hearts using enzymatic perfusion and differential ultracentrifugation. (B) Schematic outlining Langendorff-cannulation for myocardial perfusion.

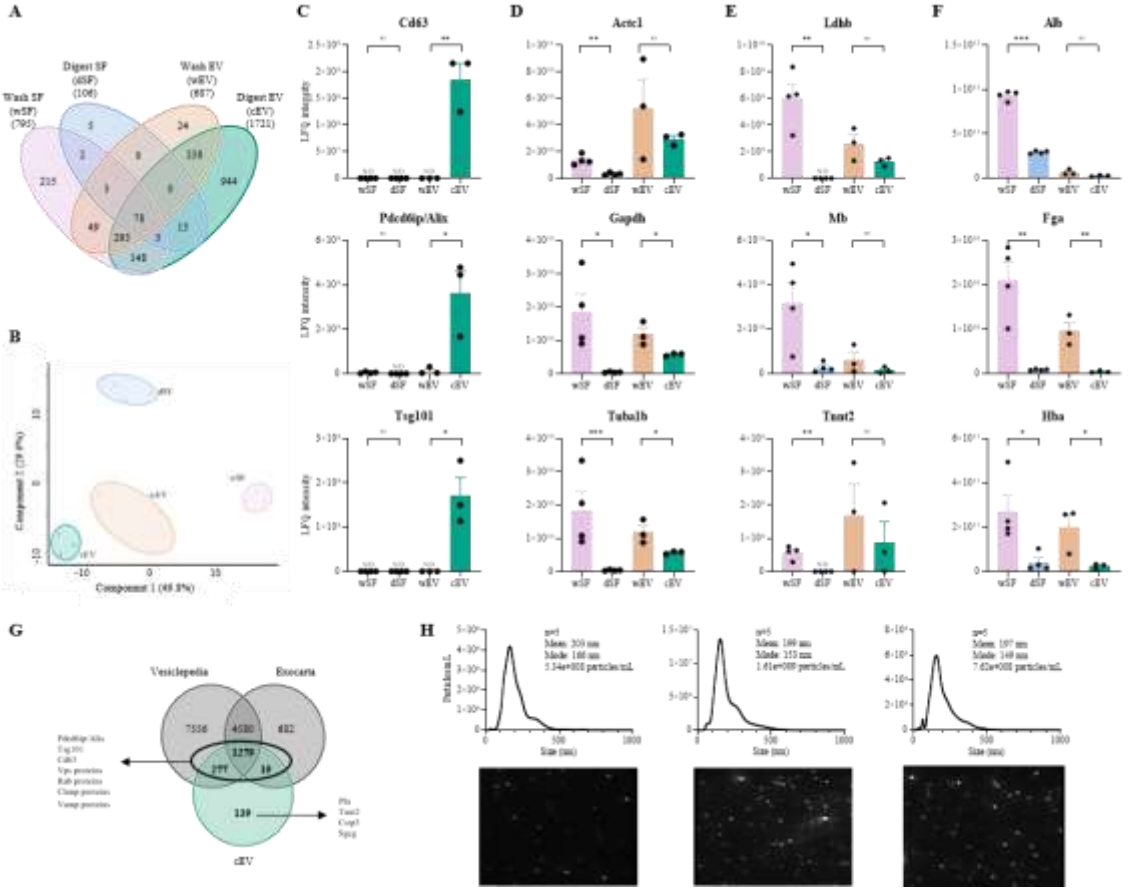

Figure 2. Characterisation of extracellular vesicles from mouse heart. (A) Venn diagram of proteins identified using mass spectrometry analysis in digest (cEV) and wash (wEV) extracellular vesicles, and digest (dSF) and wash (wSF) soluble factors. (B) Principal component analysis of cEV, wEV, dSF, and wSF. Normalised LFQ intensities of individual replicates for (C) Cd63, Pdcd6ip/Alix, Tsg101, (D) Actc1, Gapdh, Tuba1b, (E) Ldhb, Mb, Tnnt2, (F) Alb, Fga, Hba. (G) Venn diagram comparison of cEV proteome with EV proteins catalogued in Vesiclepedia and Exocarta. (H) Nanoparticle tracking analysis of cEVs (mean size ~200 nm, N = 3 biological, n =5 technical). Screen captures from video recorded using NTA. N/D = not detected, error bars = S.E.M, ns = not significant, *p < 0.05, **p <0.01, ***p <0.0001.

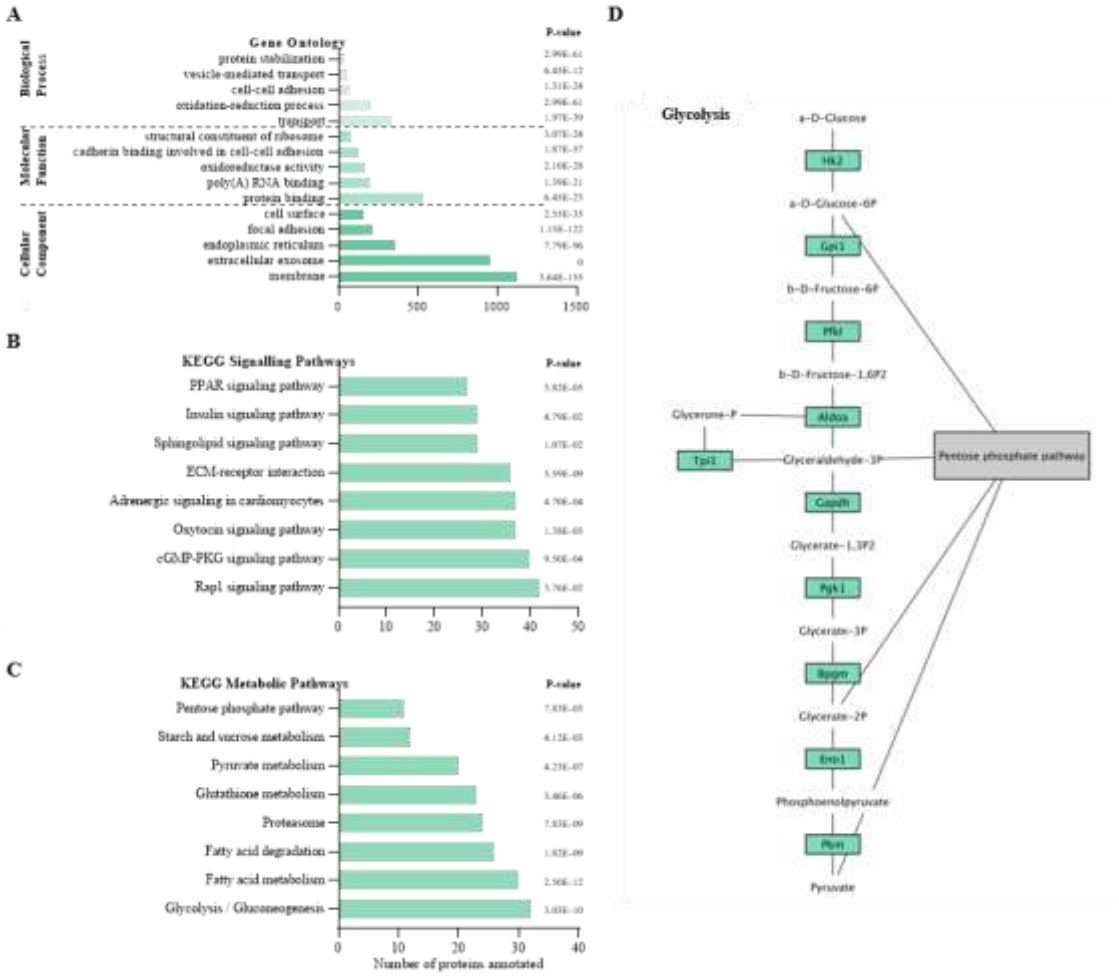

Figure 3. Bioinformatic analysis of cEV proteome. (A) Gene Ontology (GO) terms for Biological Process, Molecular Function, and Cellular Component enriched in cEV proteome. KEGG-based (B) signalling and (C) metabolic pathways enriched in cEV proteome ($p \leq 0.05$). (D) Glycolytic and pyruvate pathways (KEGG) highlighting the presence of enzymes identified in cEV (green).

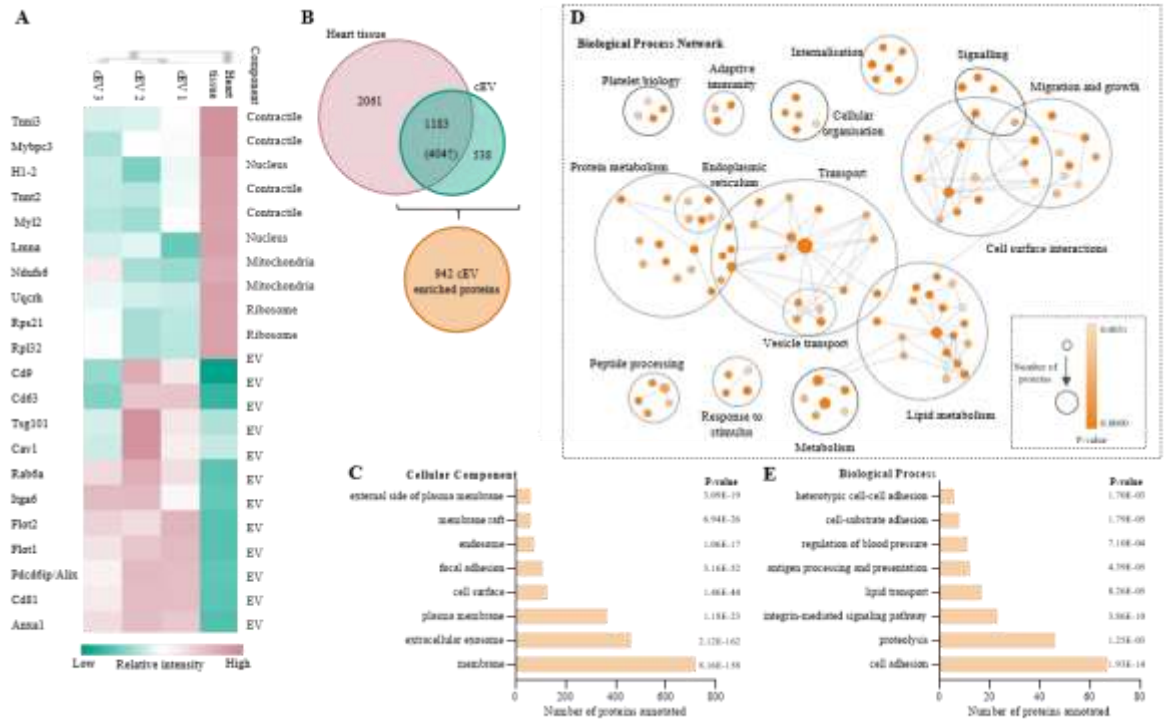

Figure 4. Comparison of cEV with cardiac proteome. (A) Hierarchical clustering of cEV proteome and heart tissue for expression of classical EV markers and cell organelle/compartment proteins; scale normalised LFQ intensity. (B) Comparison of cEV proteome with heart tissue proteome, cEV enriched proteins include proteins that are either uniquely identified or $\log_2$(fold change) ≥ 1 (in at least 2 biological replicates of cEV). (C) Cellular components enriched in cEV over heart tissue. (D) Network visualisation of GO biological processes of cEV enriched proteins using Cytoscape and EnrichmentMap ($p<0.003$). (E) biological processes enriched in cEV over heart tissue.

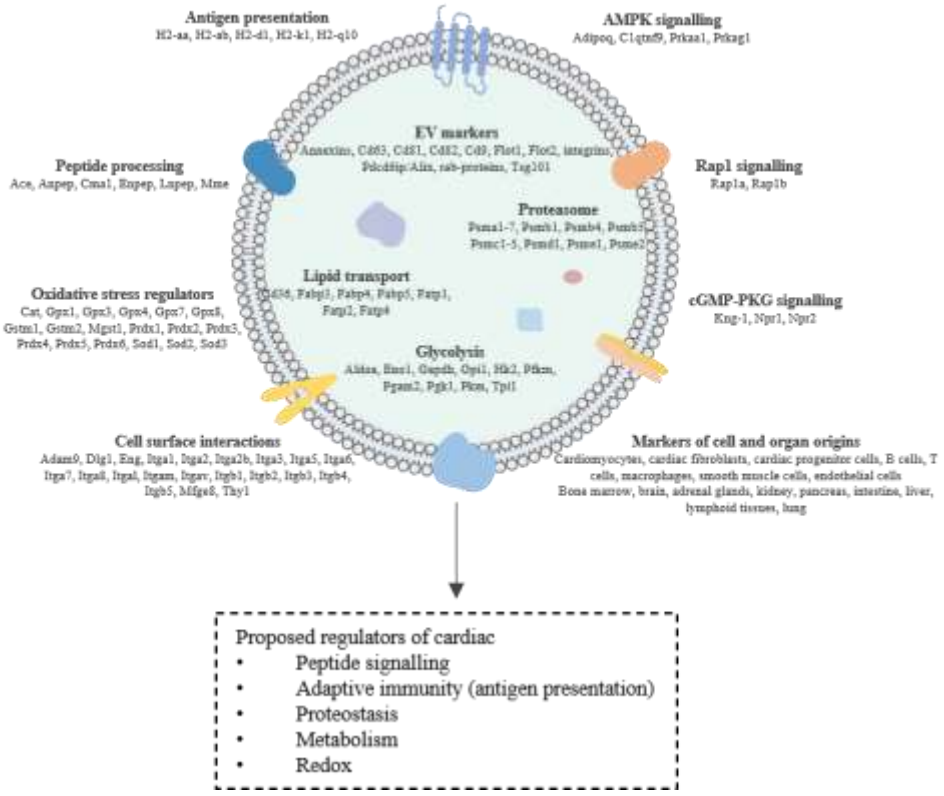

Figure 5. Summary of cEV proteomic findings. Schematic highlighting key groups of proteins present in cEVs which suggest regulatory roles in intra-cardiac and inter-organ biology.