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Review

Extracellular Vesicles in Cardiac Regeneration: Potential Applications for Tissues-on-a-Chip

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Strategies to regenerate cardiac tissue postinjury are limited and heart transplantation remains the only 'cure' for a failing heart. Extracellular vesicles (EVs), membrane-bound cell secretions important in intercellular signaling, have been shown to play a crucial role in regulating heart function. A mechanistic understanding of the role of EVs in the heart remains elusive due to the challenges in studying the native human heart. Tissue-on-a-chip platforms, comprising functional, physiologically relevant human tissue models, are an emerging technology that has yet to be fully applied to the study of EVs. In this review, we summarize recent advances in cardiac tissue-on-a-chip (CTC) platforms and discuss how they are uniquely situated to advance our understanding of EVs in cardiac disease and regeneration.

Highlights

Extracellular vesicles (EVs) play key roles in regulating cardiac physiology in health and disease.

EVs from regenerative cell sources can stimulate functional recovery of injured hearts.

Recent developments in cardiac tissue-on-a-chip (CTC) platforms have facilitated the *in vitro* replication of complex structures and functions of the adult human heart.

Advanced CTCs now allow real-time functional readouts of tissue function, incorporate multiple human induced pluripotent stem cell (iPSC)-derived cell types, and include precise structural and environmental controls, which together provide an unprecedented ability to study myocardial disease states *in vitro*.

Physiologically relevant CTCs represent effective models for the generation of new mechanistic insight into the role of EVs in cardiac function, disease, and repair, supporting the advancement of EV therapeutics towards clinical translation for treatment of heart disease.

Mechanistic Studies of Cardiac EVs Require New Model Systems

Despite therapeutic advances, **heart disease** (see Glossary) remains the leading cause of death worldwide [1]. Since the adult heart has limited ability to regenerate, injury leads to a progressive decline of cardiac function ending in **heart failure** [2]. A current lack of strategies for cardiac tissue repair and regeneration postinjury necessitates that heart transplantation remains the only 'cure' [3]. The challenges in developing regenerative therapies for the heart center on an incomplete understanding of the complex processes that underlie the physiological regulation of cardiac cells and the pathophysiology of cardiac disease progression.

Recent studies have shown that **extracellular vesicles** (**EVs**) play an integral role in regulating healthy cardiac function and that dysregulation of EVs may be an important mechanism of injury progression [4,5]. EVs are cell-secreted nanoparticles that facilitate intercellular signaling and communication by transferring **bioactive cargo**, including proteins and genetic material, to directly regulate target cells. In the heart, EV-based signaling has pleiotropic effects on disease processes including apoptosis, hypertrophy, and proliferation [6] (Figure 1A, Key Figure). Capitalizing on the ability of EVs to modulate disease, recent studies have delivered exogenous EVs therapeutically to the injured heart, resulting in improved cardiac function in both small- and large-animal models [7,8]. Despite promising results, an incomplete understanding of the mechanisms underlying EV-mediated signaling in cardiac disease and regeneration severely limits the advancement of EV-based therapies [9].

A key obstacle in the study of EVs in cardiac disease and regeneration is the lack of adequate model systems that accurately recapitulate native physiology while retaining the accessibility required to elucidate the individual mechanisms underlying EV activity [9,10]. Currently, animal models are used to best represent clinical physiology. However, animal models are not easily controllable or accessible, making it experimentally challenging to characterize the effects of EVs mechanistically. Additionally, many findings based in animal models lack translatability to human physiology. By contrast, *in vitro* human cell models are easily controllable and accessible,

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but these models lack physiological complexity and cannot faithfully recapitulate disease processes [11].

Tissue-on-a-chip platforms are a powerful emerging technology that may offer a solution. *In vitro* tissue-on-a-chip platforms integrate cells and biomaterials to mimic the structure and functionality of native organs, facilitating the investigation of human physiology in a controlled, isolated, and accessible environment [12]. These platforms can be created using human cells derived from **induced pluripotent stem cells (iPSCs)** and have already shown utility in multiple applications ranging from disease modeling to drug testing [11,12]. The use of tissue-on-a-chip platforms in the study of EVs in cardiac disease and regeneration has yet to be explored in depth but represents a promising new avenue towards the development of EV-based therapies (Figure 1B).

In this review, we describe the role of EVs in the development, progression, and treatment of heart diseases, focusing on the function of native and exogenously applied EVs. Next, we summarize recent advances in tissue-on-a-chip platforms, discussing platform capabilities and drawbacks. Finally, we offer a perspective on the application of tissue-on-a-chip platforms to the study of EVs in cardiac disease and regeneration.

EV Mechanisms of Action and Current Challenges

Biological Basis of EV Action

It has been recognized for decades that **intercellular communication** is crucial in regulating and coordinating human physiology [13]. Although first identified in the 1940s, EVs have only recently emerged as central players in intercellular communication, when studies in 2006 and 2007 linked the biological effects of EV signaling with the presence and transfer of biomolecular cargo within the EVs [14]. EVs are cell-secreted particles bound by a lipid-bilayer membrane that carry **bioactive molecules** including proteins, lipids, and nucleic acids [13]. Cells actively sort and package biomolecules from the cytosol into these vesicles which are then released into the extracellular space via exocytosis or scission of membrane buds [5,14]. To facilitate intercellular communication, EVs circulate throughout the body and bind to specific target cells via receptor–ligand interactions. They then exert a physiological response in the receptive cell either via receptor-mediated signaling pathways or through the uptake and delivery of bioactive contents into the recipient cell's cytosol [14].

EV proteins have been identified to perform roles both as surface markers for cell-specific targeting and as cytokines incorporated in the vesicle lumen [14,15]. Lipids play a critical role as constituents of the bilayer membrane that protects EV contents from degradation until they can be delivered to target cells; however the bioactivity of EV-associated lipids is currently a subject of ongoing investigation [14]. The most noted nucleic acids present in EVs that mediate their functional activity are miRNAs, small strands of noncoding RNA often implicated in the regulation of genetic expression via mRNA translational interference or degradation pathways. Through this form of genetic regulation, EV-associated miRNAs have been linked to functions ranging from modulating cellular proliferation to enhancing survival and protective responses postinjury [14–16]. Many of the mechanisms associated with the trafficking and sorting of biomolecules into EVs, triggers for EV release, and targeted action of EVs in cells and tissues remain to be defined [14].

EVs in the Heart

The heart relies on coordination between numerous specialized cell types and systems to maintain homeostasis and physiological function, making cardiac EV signaling an area of special interest. EV release and uptake by all major cell types in the heart are thought to play key roles in

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cardiac function, including regulating healthy physiology, initiating disease progression, mediating tissue repair postinjury, and coordinating multiorgan interaction processes [4,6,9,14,17–21]. Studies in the 1980s first recognized the role of EVs in cardiac pathologies, specifically in mediating the calcification of artificial heart-valve biomaterials [22]. EVs in a therapeutic context appeared in the early 2000s, initially as agents to reduce immune rejection post heart transplantation [23]. More recently, mechanistic investigations of EV signaling between cardiac cells have expanded since enhanced recognition of their significance emerged in 2007 [24]. Table 1 summarizes current knowledge surrounding the role of endogenous EV sources and their functions in the progression of myocardial disease including examples of EV signaling between the heart and other organs. Initially, EVs are involved in coordinating cardioprotective responses to limit injury, as EVs secreted by injured or immune-activated cells activate cardioprotective processes. However, the dysregulation of EV signaling in injured tissues tends to contribute to maladaptive responses including myofibroblast activation, cardiomyocyte hypertrophy, and vascular destruction, which ultimately contribute to heart failure (Table 1) [6,16,17]. Interactions between the heart and other organs in the body are known to be crucial to the coordination of systemic responses to diseases such as myocardial infarction (MI). Such interorgan signaling represents a second key role of EVs in cardiac physiology and is summarized in Table 1 [18–21].

As dysregulation of EV signaling contributes to the progression of disease, exogenous EVs isolated from **regenerative cell sources** have been analyzed for their potential in restoring function to the damaged myocardium. Recent studies have shown that applying proreparative EVs to damaged heart tissue through injection or slow-release constructs can regulate signaling processes in the body to initiate significant repair and restoration of cardiac function [4,7,16]. Exogenous isolation of EVs from cell cultures is advantageous for clinical translation due to the strict control and reproducibility of isolates, as well as the ability to manipulate culture conditions to upregulate the sorting of cardioprotective molecules into secreted vesicles [25,26]. Techniques that have been proven to amplify the therapeutic capacity of EVs from cultured cells or transform inert cells to sources of cardioprotective EVs include shear-stress and ischemic preconditioning as well as the engineering of cells to overexpress therapeutically significant genetic factors, such as GATA4 [27–30]. Therapeutic sources investigated thus far include cardiomyocytes, endothelial cells, and stem cells [16,25]. Cardiomyocyte EVs have been of particular interest, as their EVs contain the most relevant molecules for their own self-regulation, including heat shock protein (HSP)20, which has noted angiogenic, antiapoptotic, and antifibrotic properties [7,25,31]. Mesenchymal stem cell (MSC) EVs have also been the subject of much research, as they are known to mediate cardioprotective processes as well as the anti-inflammatory effects of macrophages, in part through the action of miR-223 [32,33]. Proposed cell sources and mechanisms of exogenous EV therapeutics are summarized in Table 2.

Advances and Challenges in EV Research

Although our understanding of EV signaling has advanced rapidly over the past decade, major challenges continue to hamper efficient clinical translation of EV-based therapeutics. The nascency of research surrounding EVs in cardiac signaling currently means that techniques related to EV isolation, molecular characterization, and analysis of the *in situ* mechanisms of action are often unpolished and lack widespread standardization [9]. As noted by the International Society for EVs (ISEV), there are currently no universally accepted surface markers for EVs, specifically to differentiate subtypes and sources, which makes effective purification of desired EV populations difficult [15]. While technical standardization and reproducibility represent a first step towards improving knowledge, the development of detailed mechanistic understanding of EVs in the heart represents a second and perhaps more complicated roadblock to harnessing the clinical potential of cardiac EVs.

Glossary

Bioactive cargo/molecules: biological material, such as nucleic acids, lipids, or proteins, contained in EVs that can interact with cellular components in the recipient cell to impact cellular state and/or function.
Biomaterial scaffold: a 3D network of natural or synthetic material within which cells can survive; frequently used in tissue engineering to provide structure to engineered tissues and to mimic the properties of the native ECM.
Extracellular vesicles (EVs): membrane-bound structures that are secreted by cells to deliver cargo to other cells for the facilitation of intercellular communication.
Genetically encoded sensor: a protein encoded by artificially introduced DNA that creates a recordable signal, such as fluorescence or luminescence, that changes in response to a specific stimulus or environmental condition. This change can be the result of a modification to the protein itself or to its expression.
Heart disease/heart failure: injury to the heart that results in a progressive process of irreversible organ damage until the heart is no longer able to adequately perfuse the body with blood.
Hydrogel: a specific type of biomaterial scaffold that contains hydrophilic polymers so that the material can contain large amounts of water.
Induced pluripotent stem cell (iPSC): a cell that has been reprogrammed with the artificial activation of transcription factors to reach a pluripotent state from which it can differentiate into any cell type in the body.
Intercellular communication: signals sent between nearby or distant cells that provide information to the recipient cell about the state of the donor cell.
Online readouts: indicators of engineered tissue state or function that can be recorded over time under experimental conditions without damaging the tissue.
Regenerative cell sources: exogenously cultured cells for use in regenerative medicine, either as cell-based therapy or as a source for cell-secreted therapeutics, such as EVs.
Tissue-on-a-chip: an engineered *in vitro* model of complex tissue with integrated hardware that recapitulates *in vivo* physiology more accurately than simple 2D cell culture. The term 'chip'

As *in situ* EV signaling processes are difficult to visualize *in vivo* and *in vitro* cell cultures poorly replicate human physiology, the European Society of Cardiology (ESC) and other prominent EV researchers have specifically called for 'advanced cell models' and novel 3D tissue platforms to improve our ability to investigate the mechanisms of EVs in the heart [9,10]. In researching EV therapies for treatment of the damaged heart, major limitations towards clinical approval and implementation involve poor understanding of regenerative mechanisms and their associated molecules, pharmacokinetics, safety, and the potential toxicity and immunogenicity of exogenous EVs [9,10,26]. According to the ESC, to overcome these limitations high-resolution methods of EV isolation and visualization are needed alongside improved mechanistic understanding of EVs in cardiac physiology and disease at a cellular level [9].

comes from the frequent use of microfabrication techniques originally developed for semiconductor integrated circuit ('computer chip') production in the creation of such models.

CTC Models of the Heart

At the same time that animal studies have led to discoveries about EVs, major advances have been made in the field of tissue engineering to create physiologically relevant *in vitro* models of human physiology and pathophysiology. These innovations are leading towards a paradigm shift in biomedical research, with an increased emphasis on the use of human cells and tissues to improve translational potential. Although animal research provides the foundation for modern understanding of the human body, the shortcomings of animal models are becoming increasingly clear [34]. Significant species-to-species variations are evident in biological mechanisms and contribute to the failure of most potential therapeutics to translate from success in preclinical models to improved patient outcomes [35,36].

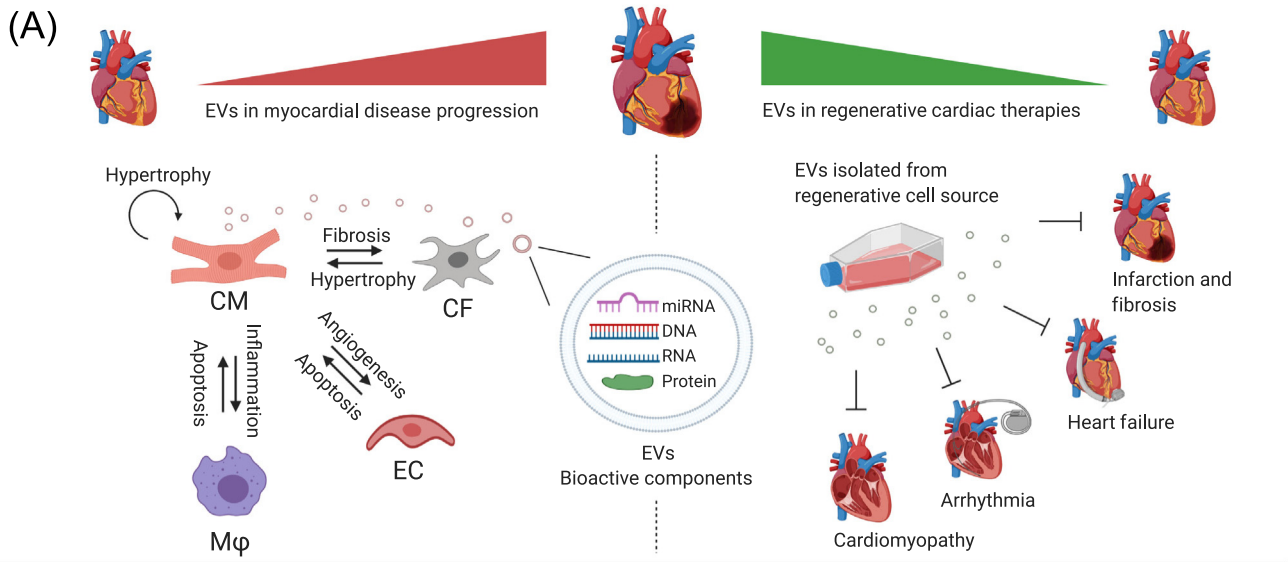
Generation of CTC Platforms

Since the 1990s, tissue engineers have developed increasingly complex and physiologically relevant models of the heart in health and disease [37–40]. CTC platforms typically include: cardiomyocytes, sometimes with additional cells, encapsulated in a **biomaterial scaffold**; supporting hardware that interacts with these encapsulated cells; and online sensors to monitor the function of the resulting engineered tissue. Common models include elongated or planar tissues suspended between pillars [41–43], planar tissues attached to flexible substrates [44,45], spheroids [46–48] or tubular tissues [49] in microwells, ring-shaped tissues looped around support posts [50–52], elongated tissues suspended along wire filaments [53], web-like tissue networks supported by geometrically patterned posts [54], and hollow constructs designed to mimic the pumping mechanism of full heart chambers [55,56]. These geometries range in size from micron to millimeter scale, depending on the device fabrication techniques used. Some platforms incorporate electrodes to electrically stimulate tissues or flexible pillars to provide dynamic resistance as tissues contract, both of which have been shown to enhance the maturation of cardiac tissues [57–60]. Platforms with dynamic medium culture conditions [49], pulsatile hemodynamic forces [61], or externally applied cyclic strain [62] have also enhanced tissue maturity and physiological function. A selection of key CTC platform designs and features are displayed in Figure 2.

Within tissues themselves, matrix components, cellular composition, and environmental factors all impact the ability of the tissue to accurately recapitulate myocardial physiology or pathophysiology. Many studies have reported that the inclusion of additional non-cardiomyocyte cell types, including dermal fibroblasts [51,58], cardiac fibroblasts [57,63,64], endothelial cells [53,63–65], MSCs [66], smooth muscle cells [53,63], or epicardial cells [67], enhance tissue function and/or cardiomyocyte maturation. A recent study that systematically characterized many of these stromal cell populations found that tissue function varied significantly depending on the phenotype of the supporting cells, suggesting that these non-cardiomyocyte cells play a critical role in engineered models [68]. With the advent of iPSC technology, most platforms use iPSC-derived

Key Figure

An Overview of the Biological and Therapeutic Roles of Extracellular Vesicles (EVs) and of the Methods for Studying EVs in the Context of Cardiac Pathophysiology



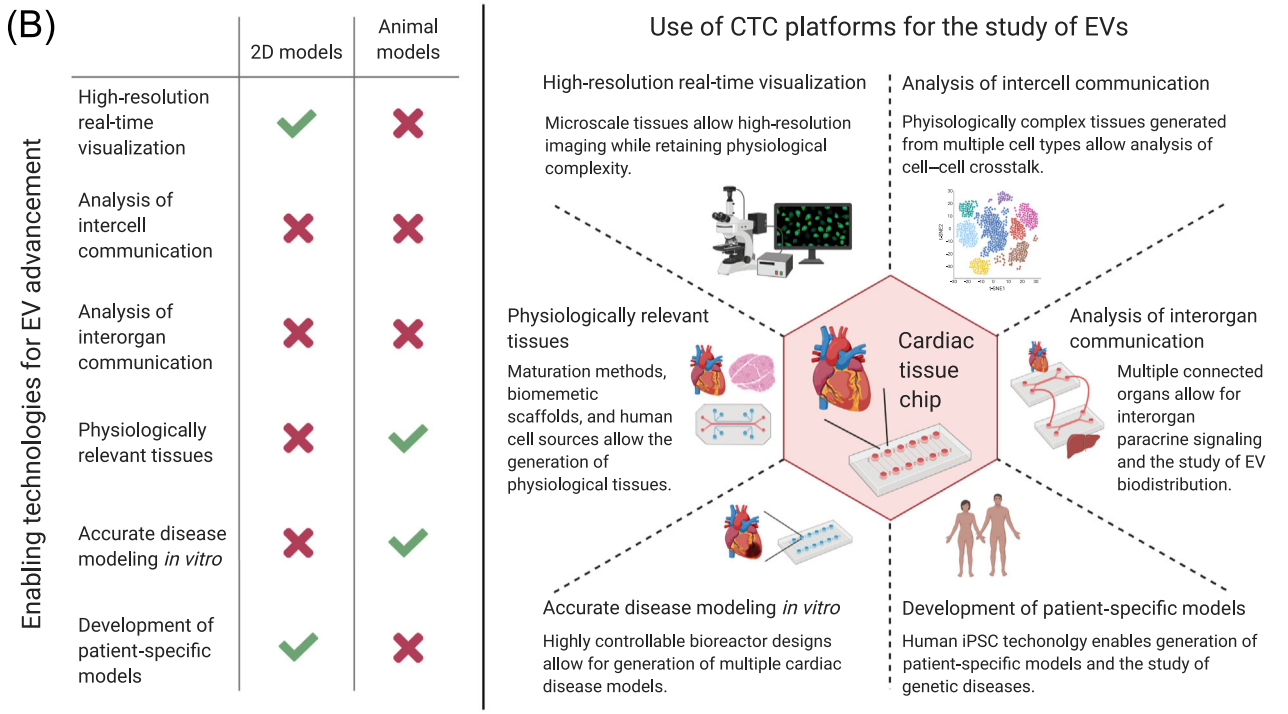


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Table 1. Summary of Known Roles and Mechanisms of Endogenous EVs in the Initiation and Progression of Common Myocardial Diseases

Roles of endogenous EVs in myocardial damage and disease progression			
Condition	EV involved, general effect	Associated molecular mechanism	Refs
MI	• Cardiac fibroblast (CF) EVs initiate cardiomyocyte (CM) hypertrophy	• miR-21-3p	[110]
	• CM EVs inhibit autophagy and favor apoptosis of nearby CMs	• miR-30a	[111]
	• CMs release large quantities of EVs with cardiac-specific miRNA as a postinjury warning signal/biomarker	• miR-1, miR-133, miR-208, miR-499	[6]
	• Macrophage EVs inhibit CF proliferation and promote inflammation, increasing risk of cardiac rupture	• miR-155	[112]
Cardiac fibrosis	• Postischemia, CM EVs enhance CF viability and promote fibrosis, fibroblast-to-myofibroblast transformation	• miR-217, miR-208a	[113,114]
	• EVs from hypertrophic CMs increase CF collagen expression	• HSP90, interleukin (IL)-6	[115]
	• Postinjury, various systemic EVs can promote CF proliferation, transformation, and collagen deposition	• TGF-β, TGF-β mRNA transcripts	[6,116]
Chronic heart failure	• Dysregulated CM and CF EVs inhibit antioxidant protein production in CM, contribute to long-term progression of tissue damage and death	• miR-27a, miR-28-3p, miR-34a	[117]
	• Plasma-derived EVs act on immune cells to contribute to chronic elevation of proinflammatory cytokines	• Elevated mitochondrial DNA in EVs	[118]
	• CM EVs promote chronic maladaptive cardiac hypertrophy and remodeling	• miR-27b, miR-155, miR-217, tumor necrosis factor (TNF)-α, miR-208a	[6,113,119–121]
Arrythmia	• CM EVs from tissues exhibiting atrial fibrillation impair ion channel and pump expression in neighboring CMs, contributing to chronic atrial remodeling and calcium handling abnormalities	• miR-208b	[122]
	• Postischemia, platelet-derived EVs may dysregulate CM calcium channels	• miR-328	[123]
Other cardiomyopathies	• Peripartum cardiomyopathy: endothelial cell (EC) EVs impair CM metabolism and inhibit angiogenic activity in ECs	• miR-146	[124]
	• Diabetic cardiomyopathy: CM EVs inhibit angiogenic activity in ECs	• miR-320; downregulation of HSP20 and miR-126	[125]
	• Septic cardiomyopathy: platelet-derived EVs induce EC apoptosis and depress CM function	• Nitric oxide synthase; downregulated miR-223	[17]
	• Dilated cardiomyopathy: CM EVs induce pathological changes to gene expression in healthy CMs, potentially have a role in adverse myocardial remodeling and disease progression	• Not defined; potential role of miR-133a	[126,127]
Roles of EV signaling between the heart and other organs/systems postinfarction			
Interaction	Brief description	Associated molecular mechanism	Refs
Heart–bone marrow	• CM EVs are preferentially imported into bone marrow where they mediate systemic injury response via downregulation of CXCR4 expression and mobilization of bone marrow progenitors into the circulation	• miR-1, miR-208, miR-499	[18]
Heart/kidney–MSCs	• EVs originating from the heart and kidneys activate proangiogenic signaling in adipose-derived MSCs as part of a postischemic reparative response	• miR-1956	[19]
Heart–monocytes	• EVs from the left ventricle, including those of CM and EC origin, induce a proinflammatory state in local monocytes as part of injury response regulation	• Not defined	[20]
Heart–spleen	• EC EVs are sequestered in the spleen where they activate and mobilize splenic monocytes, which are recruited to the damaged myocardium	• miR-126-3p and -5p	[21]

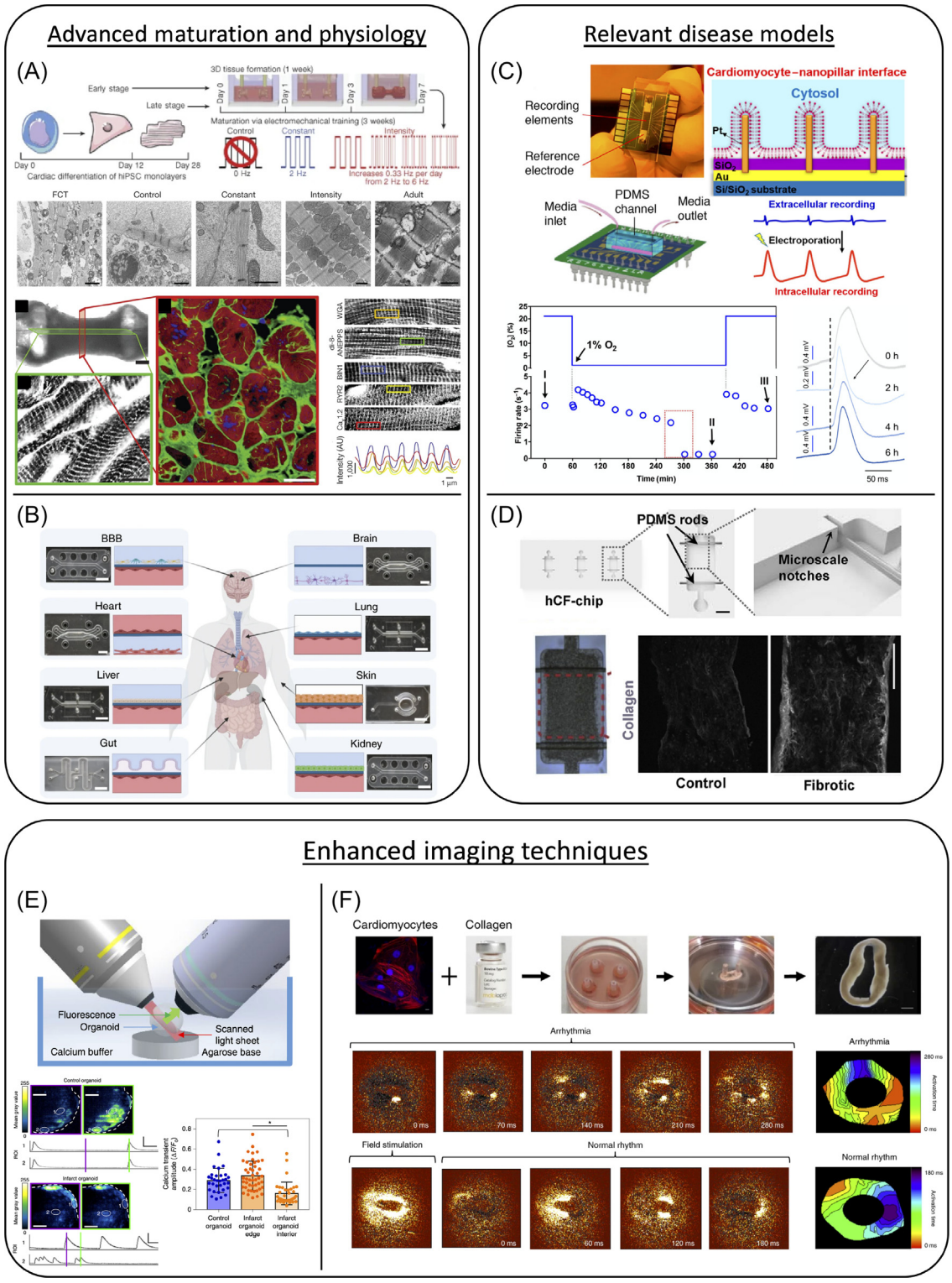
Figure 1. (A) EVs mediate intercellular communication through the delivery of cargo containing multiple bioactive components. In the heart, EVs regulate many processes critical in disease progression and are secreted from all major cell types, including cardiomyocytes (CMs), cardiac fibroblasts (CFs), endothelial cells (ECs), and macrophages. (B) Traditionally, 2D *in vitro* models and animal models have been used in the study of cardiac EVs. While effective in many applications, these approaches have significant limitations. Cardiac tissue-on-a-chip (CTC) platforms overcome many of these challenges and have the potential to revolutionize the study of EVs. Figure created with BioRender.com. Abbreviations: iPSC, induced pluripotent stem cell; Mφ, macrophage.

Table 2. Summary of Potential Roles and Mechanisms of Exogenous EVs Sourced from Cell Cultures in Mediating Cardiac Repair Postinjury

EV source	Target and potential therapeutic benefit	Associated molecular mechanism[a]	Refs
CM EVs	• ECs: promote angiogenesis via increased proliferation, migration, and tube formation	• HSP20, miR-143, miR-222; downregulation of miR-939-5p (postischemic CMs)	[31,128,129]
	• CMs: prevention of hypertrophy, antiapoptotic effects, reduced potassium channel abnormalities	• HSP20, miR-1, miR-133a, miR-499	[7,31,130–132]
	• CFs: reduction of fibrosis	• HSP20, miR-133a	[31,132]
CF EVs	• CMs: antiapoptotic, prosurvival effects	• miR-21, miR-210, miR-423-3p	[133,134]
EC EVs	• ECs: stimulate angiogenesis by repressing cell-cycle arrest; increase migration, tube formation, and sprouting	• miR-214	[135]
	• Monocytes: reduce inflammation by repressing monocyte activation to macrophages	• miR-10a	[136]
	• Vascular smooth muscle cells: inhibition of atherosclerotic lesion formation in cardiac vessels	• miR-143/145 cluster (post shear-stress preconditioning)	[27]
iPSC EVs	• CMs: significant antiapoptotic effects, improved resistance to oxidative stress, attenuation of hypertrophy	• miR-21, miR-210	[133]
	• ECs: promotion of migration and tubule formation, antiapoptotic effects	• Postulated role of miR-294, miR-16, miR-34, miR-20; proangiogenic proteins including VEGF-C, PDGFs, BMP-4, TDGF1	[137]
	• General prosurvival effects, promotion of cellular self-renewal, proliferation, improved resistance to stress	• Postulated role of miR290-295 cluster, miR-17-92 cluster, miR-126-3p, miR-130a-3p, miR-210-3p, miR145, let-7, miR-302a-5p	[137]
Embryonic Stem Cell EVs	• CMs: increased survival post-MI, potential for cell cycle re-entry	• miR-290-295 cluster, especially miR-294	[138]
MSC EVs, adipose-derived stem cell EVs	• CMs: antiapoptotic effects, preservation of mitochondrial membrane potential during ischemia; improvements in contractility and calcium handling; decreased production of inflammatory factors, fibrosis associated proteins; suppression of sustained, maladaptive autophagy	• miR-21-5p, miR-126, miR-93-5p; miR-22 (postischemic preconditioning); miR 19-a (GATA-4-overexpressing MSCs)	[28,29,109,139,140]
	• ECs: promotion of angiogenesis, increased migration, vessel formation	• miR-21, EMMPRIN glycoprotein, miR-126, miR-125a, miR-30b	[139,141–143]
	• Macrophages: anti-inflammatory signaling, promotion of proreparative macrophage polarization	• miR-223; potential role of miR-24	[32,33]
	• CFs: reduced fibroblast-to-myofibroblast transformation	• miR-22 (postischemic preconditioning); potential role of miR-29	[28,33]
Cardiac telocyte EVs	• ECs: promotion of migration, proliferation, tube formation	• Possible mediators include let-7e, miR-10a, miR-21, mi-R27b, miR-100, miR-126-3p, miR-130a, miR-143, miR-155, and miR-503	[16,144,145]
	• CFs: reduction of collagen deposition, fibrosis	• Not defined	[145]

[a]Abbreviations: BMP-4, bone morphogenetic protein-4; EMMPRIN, ECM metalloproteinase inducer; PDGF, platelet-derived growth factor; TDGF1, teratocarcinoma-derived growth factor 1; VEGF-C, vascular endothelial growth factor-C.

cardiomyocytes, and progress is being made to incorporate iPSC-derived stromal cells as well [64]. Groups have also recently begun to investigate the differing characteristics of chamber-specific cardiomyocytes for specific modeling of the ventricular or atrial myocardium [52,57]. Varying the concentrations and ratios of different cell types has also been used to promote disease phenotypes, such as modeling cardiac fibrosis by increasing the proportion of fibroblasts in tissues [69]. Environmental factors, including matrix and soluble medium composition, can also influence the ability to accurately model cardiac physiology and pathophysiology. Commonly used matrix



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components for **hydrogels** include fibrin [41,70], collagen [50], Matrigel [53], and gelatin [71] and numerous groups have attempted to optimize the concentrations and ratios of these components for specific platform functions [72]. Some groups have also eliminated the use of exogenous extracellular matrix (ECM) components and instead supported cells with synthetic fibers so that endogenously secreted matrix can be studied [73].

Key Features of CTCs

The considerations involved in the generation of CTCs also affect the types of readouts that can be measured and thus the sensors that can be incorporated. For example, planar tissues attached directly to hardware can be placed on top of multielectrode arrays (MEAs) to directly measure tissue electrophysiology [74,75] or attached to flexible strain gauges to measure contractile stress [76]. Tissues suspended between or attached to flexible supports are amenable to video analysis that can determine force generation based on the deflection and physical characteristics of the supporting material [42,77,78]. To further investigate force generation with variable tissue geometries and to model auxotonic contractions in the native myocardium, some groups have added additional hardware to manipulate the tissue on-chip [79,80]. Video analysis can also be used in combination with voltage and calcium dyes to monitor calcium transients and action potential propagation within tissues using live-imaging chambers and fluorescence microscopy, if platforms are designed with optically compatible materials and appropriate working distances. Such traditional imaging, however, is limited by the complexity and 3D depth of engineered tissues, so that it is best suited for relatively low-resolution imaging of tissue surfaces. To better investigate tissue function at single-cell resolution and in tissue constructs, groups have recently begun to incorporate live laser-confocal microscopy [81] and live light-sheet fluorescence microscopy [47,82]. Such imaging modalities have allowed single-cell action potential and calcium transient measurements, both on the surface and within engineered tissues. In addition to functional dyes, all of these microscopy platforms can be combined with **genetically encoded sensors** to continuously monitor cell function, obviating the need to add exogenous dyes or the concern about signal decay as dyes degrade. Commonly used genetically encoded sensors include the engineered calcium sensitive GCaMP protein [83] and its many derivatives and the ArcLight voltage sensor [84]. Recent studies have also incorporated sensors to measure glutathione redox state as a proxy for oxidative stress [85].

Beyond engineered tissues and their immediate environment, significant progress has been made in the incorporation of circulation connecting CTCs with endothelium, vascular networks, and other tissue and organ models. Multiple groups have successfully incorporated microfluidic channels to facilitate the mixing of media from multiple tissue-on-a-chip platforms and have demonstrated that such interorgan communication influences the physiology of the included tissues [86]. These platforms have also been leveraged to study the pharmacokinetics of drug metabolism and the distribution and impact of drug candidates on multiple organ systems [74,87,88].

Figure 2. Recent Cardiac Tissue-on-a-Chip (CTC) Platforms with Features Useful for Cardiac Extracellular Vesicle (EV) Research. (A) A pillar-based platform that uses electrical stimulation at increasing frequencies to promote the maturity of cardiac tissues comprising fibroblasts and human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes [58]. (B) An integrated 'human body-on-a-chip' platform connects a muscular thin-film cardiac tissue with seven other tissue chips via endothelialized microfluidic channels containing circulating media [93]. (C) A microfluidic chamber with environmental controls and microfabricated electrodes for intra- and extracellular recordings of action potentials during simulated myocardial ischemia with acute hypoxia [146]. (D) An engineered model of cardiac fibrosis that uses transforming growth factor beta (TGF-β) treatment of a cardiac tissue to promote a fibrotic phenotype, as demonstrated by a significant increase in collagen deposition in the fibrotic tissue [104]. (E) An engineered microtissue model of the infarcted myocardium that is compatible with light-sheet microscopy to enable measurements of calcium transients throughout the depth of the tissue [47]. (F) A ring-shaped engineered heart tissue with chamber-specific iPSC-derived cardiomyocytes in a confocal-microscopy-compatible platform. High-resolution optical mapping with confocal microscopy shows calcium transients in individual cells throughout the tissue, enabling detailed mapping of depolarization wave propagation in healthy and arrhythmic tissues [52]. For more detailed descriptions of each platform please refer to the original publications. All images reproduced, with permission, from [47,52,58,93,104,146]. Abbreviations: FCT, fetal cardiac tissue; hCF, human cardiac fibroblast; PDMS, polydimethylsiloxane.

Table 3. Recent CTC Platforms and Their Features Relevant to Investigations of Cardiac EV Signaling[a]

Name and brief description		Cell composition	Scaffold	Relevant disease model	**Online readout**	Key feature	Refs
Cardiac muscular thin films	Micromolded flexible cantilever covered with a laminar sheet of CMs	hiPSC-CMs	Gelatin	Arrhythmia (CPTV); drug-induced cardiotoxicity; cardiac hypertrophy	Contractile stress (integrated strain gauge); AP propagation (voltage dyes); Ca^{2+} transients (dye); electrophyisology (MEA)	Microfluidic; multiorgan integration with endothelial barrier; 3D printable; integrated strain gauge; optical stimulation for optogenetic cells; integrated MEA	[76,93,97,147]
Chamber-specific engineered heart tissues (EHTs)	Chamber-specific ring-shaped EHTs	hESC-VCMs or hESC-ACMs	Collagen	Arrythmia	Active force; passive tension; Ca^{2+} transients; AP propagation (voltage dyes)	Spatial mapping; chamber-specific tissues	[52]
Cardiac fibrosis-on-a-chip	Elongated microcardiac tissues suspended between horizontal PDMS rods; fibrosis induced by TGF-β activation of CFs in microtissues	hiPSC-VCMs; hiPSC-ACMs; primary CFs	Fibrin	Cardiac fibrosis	Active force; passive tension; Ca^{2+} transients	EV isolation from chip; circulating miRNA profiling	[104]
Engineered heart tissues	Elongated tissues suspended between flexible pillars	hiPSC-CMs	Fibrin	Drug-induced cardiotoxicity	Active force; passive tension; Ca^{2+} transients		[70,148,149]
Defined engineered human myocardium	Circular micromolded cardiac microtissues suspended around flexible pillars	hESC-CMs or hiPSC-CMs; human foreskin fibroblasts	Collagen	Neurohormonally induced heart failure	Active force; passive tension; optogenetic redox sensor	Advanced maturation; scalable tissues; systematic optimization of culture conditions	[51,60,85]
Human engineered cardiac tissues	Micromolded cardiac tissues suspended between PDMS posts	hESC-CMs; hESC-fibroblast-like-cells (differentiation side product); ± hMSCs	Collagen	Cardiac hypertrophy	Active force; passive tension; Ca^{2+} transients	hMSC supplementation; exogenous EV application; exogenous miRNA application	[43,105,109]

Tissue engineered model of IRI	Planar cardiac tissues suspended between four PDMS pillars	hiPSC-CMs	Collagen/fibrin	Ischemia, ischemia–reperfusion injury	Contractility	3D model of IRI	[101]
Biowire II	Chamber-specific cardiac tissues suspended between POMaC wires in an electrical stimulation chamber	hiPSC-CMs; primary CFs	Collagen/Matrigel	Left ventricular hypertrophy; cardiac fibrosis; drug-induced cardiotoxicity	Active force; passive tension; Ca^{2+} transients	Chamber-specific tissues; atrioventricular tissues; advanced maturation; non-PDMS	[57,69]
3D filamentous cardiac microtissues	CMs seeded onto a synthetic matrix comprising parallel and evenly spaced fibers	hiPSC-CMs	Synthetic filamentous matrix	Arrythmia (long QT), hypertrophic cardiomyopathy, dilated cardiomyopathy	Active force; passive tension; Ca^{2+} transients	Self-assembling without exogenous ECM	[73,98]
Intensity-trained cardiac tissues	Electromechanically trained cardiac tissues suspended between PDMS pillars in an electrical stimulation chamber	hiPSC-CMs; primary dermal fibroblasts	Fibrin	Cardiac hypertrophy	Contractility; Ca^{2+} transients	Advanced maturation; electrical maturation protocol	[58]
Cardiobundles	3D cylindrical tissues free floating in a dynamic culture system	hESC-CMs, hiPSC-CMs, or NRVMs; human CFs	Fibrin	Cardiac hypertrophy	Active force; passive tension; Ca^{2+} transients	Dynamic culture conditions	[49,150]
Biowire	Micromolded cardiac tissues suspended along a suture in an electrical stimulation chamber	hESC-CMs, fibroblasts, ECs, smooth muscle cells	Collagen	Drug-induced cardiotoxicity	Contractility; Ca^{2+} transients	Advanced maturation; incorporated electrical stimulation	[53]

[a]Abbreviations: ACM, atrial cardiomyocyte; AP, action potential; IRI, ischemia–reperfusion injury; NRVM, neonatal rat ventricular myocyte; PDMS, polydimethylsiloxane; POMaC, poly[octameythlene maleate (anhydride) citrate]; VCM, ventricular cardiomyocyte.

Although the ability to model such interorgan communication is an important milestone, many of these systems do not incorporate endothelial barriers or vasculature. Various approaches have been used to address this problem, including the addition of vascular networks to cardiac tissues [89,90], which have had moderate successes, and the placement of an endothelial barrier between a cardiac chamber containing cardiac-specific media and an additional reservoir of circulating medium [91]. The latter technique has been successfully incorporated into multiorgan models [92,93] and multiple companies are attempting to commercialize this technology alongside single-organ CTC platforms [12].

Disease Modeling Using CTCs

Alongside the development of complex, physiologically relevant CTCs has come the ability to accurately model a variety of cardiac diseases *in vitro*. Whether through the use of naturally occurring ECM proteins or synthetic polymers, the mechanics of these supporting materials can be changed to contribute to disease modeling, such as increasing matrix stiffness to model a fibrotic phenotype [94]. In addition to generic ECM hydrogels, decellularized scaffolds made from healthy and diseased animal tissue matrix have been seeded with cells to create CTCs and used to study the contribution of native ECM to healthy and diseased states, including hypertrophic cardiomyopathy [95,96]. Numerous groups have also incorporated patient-derived or genetically engineered cells to recapitulate disease phenotypes, including arrhythmias [97], hypertrophic cardiomyopathies [57,98], and dilated cardiomyopathies [98,99]. These patient-specific cell technologies have also been used to study patient susceptibility to drug-induced cardiotoxicities and patient-to-patient variability in response to pharmaceutical interventions. Groups have also altered culture medium composition to promote other phenotypes. With the prevalence of acute MI as a major contributor to morbidity and mortality worldwide, many models attempt to recapitulate loss of coronary blood flow and the resulting biochemical changes by altering the oxygen content, pH, and metabolite composition of media [100].

Due to the immaturity of iPSC-derived cardiomyocytes and their relative lack of dependence on oxygen and oxidative phosphorylation compared with adult cardiomyocytes, recapitulation of this phenomenon in CTCs has proved challenging. Recent studies, however, using enhanced metabolic or electrical maturation techniques, have demonstrated some susceptibility of CTCs to simulated ischemia and ischemia–reperfusion injury [47,101,102]. Supplementation with neurohormonal factors, such as angiotensin II or catecholamines, has been used to simulate the overstimulation of cardiomyocytes contributing to the progression to heart failure [51,103] and transforming growth factor beta (TGF-β) supplementation has been used to activate fibroblasts to model the development of cardiac fibrosis [104]. Perhaps most importantly for this review, groups have introduced conditioned media to their platforms and discovered that EVs in these media impact the function of engineered tissues [66,104–108].

This overview of current CTC platforms is not intended to be comprehensive but rather to convey the exciting progress made in CTCs and to outline the plethora of features and models available for the study of cardiac biology and pathology. A selection of recent and/or highly cited platforms that include features relevant for the study of EVs are summarized in Table 3.

Using CTC Platforms to Study EVs

As interest in cardiac EVs and the development of sophisticated CTC platforms have emerged simultaneously over the past several years, few studies have been published to date combining both of these areas [66,104,106–109]. In two separate studies, Mayourian and colleagues from the Costa laboratory combined systems biology and human engineered cardiac tissue technologies to elucidate the mechanisms underlying the therapeutic properties of human MSC (hMSC)-derived EVs [66,109]. They generated cardiac tissues by encapsulating human

embryonic stem cell (hESC)-derived cardiomyocytes in a collagen-based scaffold set between two pillars allowing measurements of contractile force in real time. By coculturing cardiac tissues with hMSCs, the authors studied the effect of hMSC-EVs on tissues, demonstrating a marked improvement in contractile force mediated by hMSC-EVs. They subsequently established that hMSC-EVs delivered miR-21, which acts on the phosphoinositide 3-kinase (PI3K) pathway, showcasing the utility of CTCs in determining EV functions and mechanisms [66,109].

Mastikhina and colleagues in the Nunes laboratory developed a cardiac fibrosis-on-a-chip model and used it to study how interstitial fibrosis affected secreted cardiac EVs and their cargo [104]. To recapitulate cardiac fibrosis, they generated tissues with human iPSC (hiPSC)-cardiomyocytes coseeded with either naive or TGF-β1-activated human cardiac fibroblasts. Comparison of fibrotic versus control tissues demonstrated both functional differences, including increased tissue stiffness and decreased force of contraction, and transcriptional differences, including elevation of fibrosis-associated genes and miRNAs. Differences were also found in the cargo of EVs isolated from fibrotic and control tissues. Interestingly, they found that miRNAs altered in fibrotic tissues were not necessarily altered in the cargo of EVs isolated from such tissues. Specifically, miR-208 was significantly elevated in fibrotic tissues but not in secreted EVs. Additionally, they found that miR-221, a known target of TGF-β signaling, was not altered in fibrotic tissues but was significantly downregulated in tissue-secreted EVs. Furthermore, treatment with the antifibrotic drug pirfenidone resulted in a larger increase of miR-221 in EVs isolated from fibrotic tissues than from the tissues themselves, suggesting a possible new mechanism for the drug's action through effects on secreted EVs. Here, biomimetic CTC disease models were utilized to investigate the potential roles of EVs in cardiac fibrosis [104]. Despite a paucity of literature, existing studies demonstrate significant potential in the use of CTCs to study the role of EVs in the heart.

Concluding Remarks and Future Perspectives

The studies discussed above provide an encouraging proof of concept for the utility of CTC platforms, laying the foundation for this exciting new intersection of tissue engineering, EV biology, and EV therapeutics. As the limitations of both 2D cell cultures and animal models for the study of EVs become increasingly clear, CTCs utilize advantages of both to fill the preclinical need for an experimentally accessible system based on realistic human physiology. As outlined in Figure 1B, CTCs leverage numerous enabling technologies, including physiologically relevant tissues, accurate disease modeling *in vitro*, patient-specific models, high-resolution real-time visualization, tissue complexity for the analysis of intercellular communication, and organ-specific tissue-chip modularity for the study of interorgan signaling. Moving forwards, we believe that these technological features afforded by CTC platforms will: (i) enable accurate recapitulation and characterization of the role EVs play in native cardiac physiology and the progression of disease; and (ii) facilitate the development of novel EV therapies by allowing the robust analysis of therapeutic EV delivery, mechanisms, and pharmacokinetics. Although challenges remain for the realization of each of these goals (see Outstanding Questions), CTCs have the potential to revolutionize the study of cardiac EVs and accelerate the translation of EV therapeutics to the cardiology clinic.

By combining complex multicellular tissue architectures and recent advances in cardiac maturation techniques, CTC platforms recapitulate cardiac physiology far more accurately than traditional 2D cell cultures while retaining the human specificity not possible with animal models. Correspondingly, this opens significant avenues of research in the study of EVs in cardiac biology and disease. Complex multicell-type tissues with the modularity and accessibility for perturbation afforded by CTC platforms will facilitate insight into the role of EVs in a broader context of cellular

Outstanding Questions

How can EV isolation and characterization from biological and cell culture samples be standardized for optimal reproducibility, yield, purity, scalability, and translational potential?

What is the role of EVs secreted by cardiac cells in the regulation of healthy heart function and what are the molecular mechanisms by which they exert these effects?

What is the role of EVs secreted by cardiac cells in the initiation and progression of myocardial disease and injury and what are the underlying mechanisms?

How can CTC platforms be most effectively used to investigate the role of EVs in cardiac physiology, disease, and regenerative therapeutics?

How can benchmarking be used to relate information regarding cardiac EV signaling in CTC platforms *in vitro* towards improved understanding of cardiac EVs *in vivo*?

How can structural and environmental controls, as well as the utilization of patient-specific cells, be used to create highly relevant models of a variety of myocardial diseases *in vitro*?

Which sources of exogenous EVs are most effective at restoring function to damaged and diseased hearts and what are the mechanisms of EV-mediated cardiac repair?

How can CTCs be used to produce key data to expedite the translation of EV-based cardiac therapeutics, specifically concerning EV delivery, pharmacokinetics, and biodistribution?

What are the roles and mechanisms of EVs in physiological and pathophysiological signaling between the heart and other organs in the body?

How can CTC platforms be integrated into body-on-a-chip platforms to study interorgan EV signaling?

How can the physiological relevance of existing CTC platforms be enhanced to better recapitulate EV signaling in the native myocardium?

communication, since EVs *in vivo* interact with stromal cells, immune cells, and other supporting cells to influence cardiac pathologies and repair. To realize this potential, researchers must first comprehensively characterize the EVs secreted by CTCs and compare them with known benchmarks present in native cardiac physiology. High-dimensional data collection, including omics technologies and multiparameter functional assessments, coupled with clustering techniques are likely to improve the validity of such benchmarking and increase confidence in the relevance of CTC models.

How can the maturity of *in vitro* cardiac tissues be improved further to more closely replicate the phenotype of adult myocardium?

How can structural tissue engineering, particularly engineered vasculature, be used to improve the physiological relevance of CTCs?

Parallel to the advantages they bring to the study of cardiac EV signaling by recapitulating native physiology, CTCs are also uniquely situated to advance studies of EV-based therapies through accurate modeling of disease processes *in vitro*. Particular challenges hindering the clinical translation of EV-based therapeutics surround understanding of their pharmacokinetics and biodistribution. The *in vivo* environment presents numerous practical challenges to the accurate tracking of EVs and determining whether they reach the desired target cell, but CTC platforms overcome many of these issues. The accessibility afforded by CTCs means that the kinetics of EV uptake can be closely monitored in real time alongside dynamic quantifications of tissue responses for various dosing and delivery regimes. With the recent developments of functional vasculature in engineered tissues with robust endothelium, it becomes possible to study how EVs leave the bloodstream and enter the tissues. EV injections can be replicated *in vitro* by resuspending harvested EVs in CTC culture medium, while intravenous delivery and action can be modeled by adding EVs to perfusate flowing through engineered vessels in vascularized platforms. Encapsulation of EVs in time-release scaffolds is another potential mode of clinical delivery that can be assessed *in vitro* by placing EV-loaded constructs in close proximity to tissues in culture media. Additionally, tissues can even be dissociated and the cells sorted to study the differential uptake of EVs by different cell types. Thus, performing such experiments in CTCs can produce important translational data to expedite the clinical implementation of EV therapies.

Looking beyond the recapitulation of single tissues, multiorgan platforms will also significantly benefit the advancement of our understanding of EVs. The ability to interconnect multiple physiologically relevant tissues through functional vasculature combined with advances in real-time imaging and superior accessibility will elucidate the ways by which EVs facilitate intercellular communications not just within a specific organ, but systemically between organs. Box 1 provides more in-depth discussion of body-on-a-chip platforms.

Despite tremendous progress, challenges remain to create even more accurate models of the heart, especially in the context of disease. As the field continues to develop more sophisticated modalities for tissue maturation, approaches integrating multiple modalities will be likely to lead to more mature cells and tissues that more accurately represent healthy and diseased phenotypes. Incorporating engineered vasculature remains an ongoing challenge, but is also likely to contribute to improvements in such accuracy given the highly vascularized nature of the native myocardium. Optimizing scaffold materials, fabrication, and cell seeding in artificial vasculature are all key to the creation of robust vascularized constructs that replicate *in vivo* vessel functionality.

When developing more advanced CTC platforms, however, care must be taken to consider the tradeoffs of cost, throughput, and scale. While more complex systems may be more accurate, they are generally more difficult and expensive to scale, so appropriate systems must be chosen for the experimental question in mind. Initial screening studies could be performed in 2D cultures or simpler high-throughput systems, while in-depth validation or physiological studies could utilize more complex and accurate platforms. To ensure that responses seen in CTCs accurately mimic

Box 1. EVs and Body-on-a-Chip Models

The increasing sophistication of interconnected chips that incorporate multiple tissue types to create 'body-on-a-chip' models provides a powerful tool for the study of EV biology and the testing of EV therapeutics that remains to be utilized. Animal studies are the current gold standard for such investigations of EVs, but they are notoriously low throughput, expensive, laborious to genetically perturb, and ill-suited for high-resolution tracking of EVs and, perhaps most importantly, they do not accurately recapitulate human physiology. Body-on-a-chip platforms, such as those recently developed by the Ingber laboratory and highlighted in Figure 2, recapitulate this human physiology at the tissue level while allowing interorgan communication through endothelial barriers, all in an experimentally accessible format.

We believe that this technological advance enables a set of experiments that could dramatically increase our knowledge of EV signaling in the human body, beyond just the heart. EVs in each tissue compartment could be profiled and the function of each tissue measured with a combinatorial set of connected or disconnected tissue chips. This would enable investigators to evaluate the presence of EVs secreted from various tissues in the interstitium or cell fraction of a particular tissue in question and then determine the effects of these different EVs on tissue function. The cells in individual tissues could then be genetically or pharmacologically perturbed to assess the effects of targeted manipulations or disease models on such EV distributions. These studies would be likely to shed light on the biological contributions and mechanisms of EVs in interorgan communication. Genetic engineering to label EVs secreted from specific cells would facilitate real-time tracking of EVs as they move between tissue compartments and interact with specific target cells. Manipulations of source and/or target cells could provide insight into mechanisms of EV homing and sorting that could inform the development of EV-based therapeutics to target specific cell types. Potential therapeutics could then be tested in such body-on-a-chip models to assess the efficacy of targeting and the potential for off-target effects.

We acknowledge that model validation will be needed for all of these experiments, with benchmarking to clinical human and preclinical animal data, but this *in vitro* platform provides scale and accessibility for manipulation that would not be possible in clinical or animal studies. As with many model systems, general validation of a body-on-a-chip could be conducted and then screening could be completed at scale before hits or results are confirmed with additional models.

those *in vivo*, rigorous benchmarking with clinical and preclinical animal data for healthy and diseased states will be crucial. After these issues in accuracy and benchmarking are addressed, we believe that CTCs will play an important role in building mechanistic understanding of cardiac EVs and testing EV therapeutics, dramatically improving the utility of accessible *in vitro* systems while eliminating the species-to-species variability so often seen in animal studies.

We have given a perspective on the potential of tissue-on-a-chip platforms for studying EVs in cardiac disease and regeneration. Mechanistic understanding of the role of EVs in cardiac physiology and pathophysiology remains limited due to poor access to the heart *in vivo* and inherent physiological differences in animal versus human physiology. Thus, scale-up and clinical approval towards the translation of cardiac EV therapies have been delayed. The application of human CTC platforms in studies of cardiac EVs would offer numerous advantages due to their physiological relevance, accessibility, incorporation of built-in readouts, control over environmental parameters, and ability to replicate disease and interorgan signaling. Tissue-on-a-chip models of EVs in cardiac disease and regeneration represent a promising new avenue towards the future development of EV-based therapies that will revolutionize quality of life and outcomes for heart patients.

Author Contributions

K.T.W., T.R.N., B.L., G.V-N., and M.R. conceptualized, outlined, and edited the manuscript. K.T.W., T.R.N., and B.L. surveyed relevant literature and wrote the manuscript.

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