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"Cardiac extracellular matrix proteomics: challenges, techniques, and clinical implications"

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Abstract

Extracellular matrix (ECM) has emerged as a dynamic tissue component, providing not only structural support, but also functionally participating in a wide range of signaling events during development, injury and disease remodeling. Investigation of dynamic changes in cardiac ECM proteome is challenging due to the relative insolubility of ECM proteins, which results from their macromolecular nature, extensive post-translational modifications and tendency to form protein complexes. Finally, the relative abundance of cellular and mitochondrial proteins in cardiac tissue further complicates cardiac ECM proteomic approaches. Recent developments of various techniques to enrich and analyze ECM proteins are playing a major role in overcoming these challenges. Application of cardiac ECM proteomics in disease tissues can further provide spatial and temporal information relevant to disease diagnosis, prognosis, treatment and engineering of therapeutic candidates for cardiac repair and regeneration.

Keywords

cardiac diseases; decellularization; extracellular matrix biomarker; extracellular proteomics; extracellular matrix remodeling

1. Importance of ECM proteins

1.1 Cardiac ECM structure and function

Extracellular matrix (ECM) is a structurally supportive scaffold that governs cellular functions, such as growth, metabolism, survival and differentiation, through the coordination of spatiotemporal signal transduction and mechanosensitive cellular adhesion [1, 2]. ECM is organized into a complex network of fibrillary proteins, elastic fibers, glycosaminoglycans, glycoproteins, and adhesive proteoglycans that impart biochemical and biomechanical properties to inform cellular and tissue functions [1-3]. Additionally, due to the dynamic

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functional properties of cardiac muscle, structural and functional properties of cardiac extracellular matrix (cECM) are critical in maintaining myocardial systolic and diastolic functions [4]. The architecture and composition of cECM are subject to continuous cell-mediated turnover, allowing for dynamic remodeling of the cECM niche. In turn, the cECM niche plays a critical role in determining the interaction and response of cells with their dynamic microenvironment. Consequently, ongoing cell-matrix and matrix-cell interactions delineate the biophysical associations that govern cellular organization, fate and ultimately functional properties of myocardial tissue [2, 4].

Both cECM and myocardial cell populations are heterogeneously distributed throughout the heart. Two-thirds of myocardial tissue is comprised of cardiac fibroblasts, whereas two-thirds of the total tissue volume is comprised of specialized atrial and ventricular myocytes along with specialized conduction system. The remainder of cardiac tissue is comprised of endothelial cells, specialized cells of the heart valves and vascular smooth muscle cells [5]. Complex interactions between these cell types and their respective ECM mediate biomechanical communication involved in mechanotransduction as well as substrate stiffness, elasticity, and stress [5]. In cardiomyocytes for instance, cECM proteins contribute to a collagen-integrin-cytoskeleton-myofibril link that aligns myofibrils to assist with transmit myocardial stress and mechanosensory cues, thereby modulating protein synthesis, biochemical signaling and cellular remodeling [6]. cECM composition is classified into three categories: (1) structural proteins consisting of fibrillar molecules (e.g, collagen types I and III), (2) basement membrane (pericellular) proteins that interface between the interstitium and cardiomyocytes, and (3) nonstructural (interstitial) proteins such as proteoglycans, glycosaminoglycans, glycoproteins and non-proteoglycans [6]. Collagen types I and III comprise 10-15% of cECM, providing tensile strength and extensibility [5, 6]. The basement membrane consists of proteins, such as laminin, fibrillin, and collagen type IV. Lastly interstitial proteins, such as fibronectin, cell surface proteoglycans, hyalectans, basement membrane proteoglycans, and small leucine rich proteoglycans, are nonstructural proteins that bind to and sequester growth factors and cytokines, which can subsequently be released in response to cellular signaling cues [5, 6]. The cECM niche therefore exhibits a complex hierarchical structure with heterogeneous distribution of its constituent components and resultant spatial distribution of its functional elements.

In addition to structural components, cECM also contains growth factors that further modulate cellular function and protease activity, thereby regulating ECM biosynthesis and breakdown. Vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and transforming growth factor β (TGF- β) are all growth factor examples, which are vital to cellular development and differentiation [3, 7]. Such growth factors are heterogeneously distributed throughout cECM and often sequestered by binding to fibrillar cECM components, to serve as a deposit for release following tissue injury [2, 7]. For example, both FGF and VEGF keenly bind to heparan sulfate, a constituent of numerous cECM proteoglycans [8]. Additionally, VEGF and other growth factors are also able to bind directly to other cECM proteins. Specifically, VEGF binds to fibronectin type III domains found in tenascin-C [8]. Consequently, the distribution of various growth factors throughout the cECM is dependent on both cellular production of the individual factor and availability of their binding partners. Matrix metalloproteinases (MMPs) are cell secreted protease

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enzymes that degrade and remodel the cECM when a surge in growth factors and cytokine activity is elicited by mechanical stimulation or tissue injury leading to wound repair [9]. Since either excessive or insufficient cECM degradation is deleterious to tissue remodeling and function, MMPs are tightly regulated at transcription and quiescent until activated [9]. Finally, tissue inhibitors of metalloproteinases (TIMPs) are used to regulate MMP activity and prevent excessive matrix degradation [2, 3, 9]. The ability to characterize cECM structural and functional changes and elucidate their regulatory response through growth factors, MMP and TIMP activity, is crucial in establishing a comprehensive characterization and function of the cECM throughout development and disease.

1.2 Cell-matrix and matrix-cell interaction and signaling

Cell function and behavior are influenced by both signaling molecules and ECM adhesive interactions, both of which are capable of triggering cell differentiation and remodeling [10]. As previously discussed, cECM can act as a reservoir for growth factors and other cytokines that are released into the microenvironment by MMP activity and matrix degradation [2, 3, 9]. When bound to growth factors, ECM proteins can regulate the spatiotemporal gradients of factors that activate cell development and patterning [11]. Growth factors in the FGF receptor and TGF-β families provide examples of the complexities of cell-matrix and matrix-cell signaling. FGFs are secreted into the ECM and bind to glycosaminoglycans and FGF receptors, relaying signals pertaining to scar formation, cellular differentiation and migration [3, 12, 13]. Similarly, when bound to decorin or biglycan, TGF-β1 can be stored as a reservoir in cECM. As a result of injury, TGF-β1 can dissociate from its binding partners and become a potent activator of fibrosis through myofibroblasts activation and increased expression of type I and III collagen, proteoglycans, contractile proteins, cytokines, fibronectin and other proteins that influence cell cycle and cell fate [3, 6, 11, 13]. Consequently, ECM structure and composition combined with the spatiotemporal distribution of growth factors, MMPs and TIMPs, result in complex dynamic cell-matrix and matrix-cell interactions that collectively inform tissue function, remodeling, repair or scar formation.

In addition to acting as a reservoir for growth factor release, ECM also moderates cellular biochemical pathways through direct interactions of cell surface receptors with cECM structural components [11]. Cell-matrix interactions are enabled by specific cell surface receptors, such as integrins and cadherins. They mediate cell-cell and cell-matrix signaling and are dynamic mechanosensitive assemblies that change and respond to both physical and chemical cues [14]. Once bound, integrins and associated cytosolic proteins undergo activation, forming focal adhesions to effectively link cECM to the cellular cytoskeleton [6, 14]. Together, integrins and focal adhesion induce cell differentiation and proliferation, transduce force generation from actomyosin to cECM, and regulate cytoskeleton organization and physical interaction [14]. Integrins are able to achieve their “outside-in” and “inside-out” signaling roles through dynamic conformational changes in their head and tail domains [3]. Therefore through reciprocal communication with cECM, resident cells can alter their phenotype, building and reshaping their surrounding matrix through deposition and degradation of cECM components to adapt to the constantly changing tissue microenvironment [3, 9, 11, 13, 15].

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Dysregulation and remodeling of cECM has been implicated as a major mediator of pathological conditions [10]. For example, cardiac stress caused by myocardial infarction (MI) leads to increases in tissue stiffness and wall stress due to fibroblasts undergoing cellular transformation into alpha smooth muscle actin positive myofibroblasts, which promote cECM overexpansion [5]. Mechanical load in the cECM is sensed by integrins and results in promotion of myofibroblast matrix deposition and maintenance through activation of the TGF-β pathway [12]. As the matrix becomes more rigid, myofibroblasts are increasingly activated leading to further matrix deposition and establishment of a feed-forward loop linking matrix architecture to cell phenotype and function [12, 16]. Such changes induce systolic and diastolic dysfunction, causing abnormal biomechanics that contribute to heart failure [5, 6]. Such mechanotransduction is fundamental in both stress adaptation and tissue repair, which are primarily modulated by cECM and cellular focal adhesion interactions to induce cytoskeleton remodeling and alterations in cellular function [10-12, 14]. The use of proteomics as a tool to provide insight to ECM characterization and architecture is instrumental in understanding disease progression through cECM remodeling, cell-matrix interactions and signaling.

2. Challenges of ECM proteomics

We primarily focus our discussion here on ECM proteomics approaches and studies pertinent to cardiac diseases due to their broad clinical impact and need for improved therapeutic designs. Sample prefractionation is a mainstay of proteomic discovery, due to the advantages of reducing sample complexity and minimizing solubility incompatibles between proteins of differing physiochemical properties. Traditional global proteomics approaches favor extraction of cytoplasmic, membrane, mitochondrial and nuclear proteins using mild to moderate ionic and non-ionic detergents [17]. However, these subcellular fractionations largely fail to encompass ECM proteins, which are predominantly left behind in the insoluble pellet fraction. While compartmentalized cellular proteomics gives insight into the relative distribution of proteins within cells, and has undoubtedly provided important discoveries [17], a more comprehensive approach to whole tissue proteomics remains lacking.

Although recent studies have substantiated the critical roles of ECM in tissue development, injury and repair, functional ECM proteomics remains a challenge for numerous reasons. Firstly, ECM proteins are present in relatively low abundance when compared to cellular or mitochondrial tissue components. Secondly, ECM proteins tend to form complex macromolecular structures with low solubility in aqueous solutions. Finally, ECM proteins are subject to a wide variety of PTMs, which further increases their proteomic complexity.

2.1 Low ECM protein abundance

Compared to cellular and mitochondrial proteins, ECM proteins are present in relative low abundance (~1% of proteome) [1]. Low abundance of ECM proteins reduces the likelihood of their detection when using whole tissue homogenates, as they are overwhelmed by more abundant cellular, nuclear and mitochondrial proteins [1]. This is particularly critical in tissues with highly organized ECM and high metabolic activity, such as myocardial tissue, where highly abundant cellular proteins (e.g., actin, myosin heavy chains, titin) and high

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mitochondrial content can easily mask the present of low abundant ECM proteins following whole tissue homogenization [18]. Consequently, prefractionation techniques, which focus on removing cellular proteins, are required to effectively concentrate ECM proteins in the resultant pellet allowing for later ECM protein solubilization and proteomic analysis [19-21].

2.2 ECM protein complexes and insolubility

Many ECM proteins form macromolecular structures in their mature form, and this contributes to their inability to be solubilized during cellular fractionation. Fibrillar type collagen I molecules for instance, which represent a universal ECM component, are staggered to form triple helical collagen (procollagen) [22]. Once secreted, the procollagen is then cleaved by specific proteases to remove its N- and C-propeptides to form tropocollagen and then assemble into large macromolecular complexes in the extracellular space. This highly complex fibrillary organizational structure is then further modified through pyridinoline crosslinks and addition of collagen binding proteins or other collagen types [23]. The hierarchical assembly of collagen molecules constitutes a heterogeneous macromolecular complex of collagen fibrils to provide structural stability and characteristically makes collagen highly insoluble in most tissues [24]. Even with prefractionation technique to enrich for ECM proteins, the fibrillar protein complex remains insoluble. Recent proteomic studies have employed protease digest (trypsin) as a key component to disrupt such large protein complexes into smaller fragments, which can enhance their solubility when subjected to various solubilization techniques [19]. Additionally, stepwise ECM protein extractions of whole tissue solubilizing newly synthesized ECM proteins via sodium chloride (NaCl), removal of cellular proteins via sodium dodecyl sulfate (SDS) decellularization and solubilization of mature ECM proteins via guanidine hydrochloride (GuHCl) has facilitated the identification of novel ECM proteins critical to cardiac tissues and diseases [18, 20, 25, 26].

2.3 Post-translational modifications of ECM

ECM proteins undergo extensive PTMs, such as glycosylation, phosphorylation, acetylation, ubiquitination, and crosslinking to achieve their intricate mature configurations. All nascent proteins are subjected to modulations by PTMs, which builds in layers of complexity and flexibility to allow proteins to be precisely targeted to various sub-cellular compartments and ultimate functions. The abundance of PTMs in ECM proteins further increases the tendency of ECM components to form aggregates and macromolecular complexes reducing their solubility in aqueous environments. Since PTM's alter the physiochemical properties of the protein, such modifications can complicate analysis of the protein under proteomic conditions. Critically, if the PTM is physiologically relevant, then specialized proteomic methods are essential to detect the presence or absence of the PTMs. For instance, Lundby et al. applied specialized solubilization and enrichment technique (via titanium oxide) to facilitate detection of the proteins that are phosphorylated during cardiac β-adrenergic signaling and identify specific phosphopeptide targets [19]. Mouse whole heart tissues were homogenized and digested with trypsin followed by titanium oxide enrichment for phosphopeptides. Phosphopeptides were analyzed via LC-MS/MS, allowing identification of 670 site-specific phosphorylation changes as response to *in vivo* β-adrenergic signaling.

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Similar approach can conceptually be applied to the PTMs of cECM proteins. Glycosylation represents another common modification of ECM proteins during synthesis to enable their function and localization. Recent application of glycoproteomics has significantly enhanced our understanding of ECM modulation in cardiac function. Tian et al. enriched N-glycopeptides via hydrazide SPE from MI samples to identify the nature of these ECM proteins (transmembrane or secreted) and further quantify the relative glycosylation site per protein [27]. This study provided the novel information on *in vivo* number of N-glycosylation sites and their topology, which can be informative for drug targets and biomarker development. Proteomic investigation of specific PTMs that are differentially regulated during tissue injury and repair may help elucidate molecular mechanisms of disease.

2.4 ECM turnover in tissue development and disease

ECM is dynamically remodeled and regulated by cell-matrix interactions, therefore investigation of ECM protein spatial distribution and temporal changes is important to furthering our understanding of tissue development and disease remodeling. Functional impact of ECM proteins on tissue maturation, development and disease is evident in several pathological conditions attributed to ECM protein mutations. For example, glycine substitutions in collagen I inhibit triple helix folding of this tensile protein and leads to procollagen aggregation within the endoplasmic reticulum [2, 16]. This aggregation is responsible for the brittle bone development of osteogenesis imperfecta (OI) and illustrates the importance of correct ECM biosynthesis [2, 16]. Marfan syndrome is another autosomal dominant disorder of the ECM characterized by aortic dilatation and dissection, ectopia lentis, and long bone overgrowth with joint laxity. Mutations in the fibrillin-1 gene reduce extracellular microfibril growth and excess growth factors (i.e. TGF-β) stored in the ECM are released [28, 29]. Identification of the causal ECM protein mutations responsible for Marfan syndrome (fibrillin-1) and its association with TGF-β signaling pathway involved in ECM biosynthesis provided a deeper molecular understanding of the disease. This understanding led to subsequent studies with targeted mechanism of action via TGF-β signaling blockade, which demonstrated reduction in aortic defects in mice and human patients [28, 30]. Fibrotic diseases, like pulmonary fibrosis and ischemic cardiomyopathy (ICM), are characterized by abnormal deposition of ECM resulting in impairment of tissue functional capacity. This replacement fibrosis is usually associated with subsequent organ stiffening followed by an eventual loss of tissue function [8, 12, 16]. As the matrix becomes more rigid, myofibroblast are also activated leading to further matrix deposition and a feed-forward loop linking matrix architecture to cell phenotype is established [12, 16]. These examples showcase the critical importance of ECM biosynthesis at modulating healthy tissue development. Although somewhat successful, previous attempts to identify disease related ECM protein abnormalities have utilized static assessment of individual proteins, which fails to capture the dynamic nature and compositional complexity of the ECM in health and disease.

2.5 ECM proteomics approaches

Current cECM proteomic approaches have utilized a variety of methods to investigate both composition and function of the ECM: 1) unfractionated whole tissue or tissue biopsies from

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healthy or diseased host, 2) ECM molecules isolated or enriched from tissues, 3) cell culture-derived ECM components obtained from primary cell types, stem cells or immortalized cell lines to study the microenvironment associated with the cell of interest (Figure. 1 and Table 1). Each approach provides valuable knowledge toward reciprocal cell-matrix interactions and offers complementary data regarding ECM formation, composition and turnover in healthy and diseased states. For proteomics involving whole tissues, ECM samples typically undergo homogenization, fractionation and solubilization prior to analysis using MS-based identification. Recently, cECM tissue proteomics has benefited from a focus on ECM enrichment strategies via decellularization, sequential solubilization and extraction of ECM constituents, which has expanded the coverage and resolution of ECM proteomic investigations [18, 20, 26, 31]. Secretome analysis of various cell types can inform newly synthesized ECM proteins *in vitro* and facilitates understanding of their respective *in vivo* functions in both health and disease. Quantitative iTRAQ and SILAC labeling have recently been utilized to enable relative quantification of newly synthesized ECM proteins [32-34]. These approaches highlight the novel application of proteomics in identifying previously unknown candidates involved in the key regulation of cell behaviors and interactions with the ECM niche. A summary of cardiac ECM proteomics examples discussed in this review is listed in Table 1.

3. Motivation and approach to ECM proteomics in clinical applications

ECM proteomics has potential application to a wide range of clinical scenarios including biomarker discovery, monitoring of disease progression and therapeutic target discovery. Dynamic remodeling and interdependency of cell-matrix interactions result in the ECM being intimately involved in pathogenesis of cardiac diseases, such as ICM, the leading cause of heart failure in the United States [35]. In ICM, multiple ischemia/reperfusion (I/R) events trigger an organ level response involving myocytes death, myofibroblast and inflammatory cell activation, and ECM remodeling. Because myocardium has an intimate relationship with the circulation, protein sampling of circulating necrotic byproducts (i.e. myoglobin, creatine kinase and troponins, etc.) have made establishing a diagnosis in clinical medicine a routine matter [36]. However, remarkable challenges still remain for risk stratification, selection of therapies and monitoring patient response to therapy [37]. In ICM patients, the cardiac remodeling that leads to terminal organ failure is smoldering and heterogeneous over time. Tissue pathology may also be localized and patchy in distribution throughout the left ventricle (LV). This natural progression of disease makes it difficult to determine the ideal time to sample blood and/or tissue, and how often re-sampling should occur. Unlike oncological diseases where tumors and blood can be readily sampled at the same time, cardiac biopsies are not part of the routine care of patients with ICM [37]. When a cardiac biopsy does occur, it is usually taken from the right ventricular septum because of clinical feasibility, while the primary disease manifests in the LV. Hence, the challenge of sampling the ECM proteome in the blood of patients with ICM arises from the lack of concomitant tissue sampling to understand and validate circulating biomarkers and disease progression.

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3.1 ECM proteomics on biomarker discovery

The combination of dynamic remodeling and involvement in disease pathogenesis makes ECM a prime target for biomarker discovery. The propensity for ECM constituents to undergo pathologic dynamic remodeling results in alterations in cellular production of secreted ECM components and their breakdown products. In general, ECM secreted precursors and breakdown products exhibit greater solubility than the remaining macromolecular, fibrillar ECM components, which increases their availability for proteomic analysis. Finally, as discussed above due to the challenges involved in obtaining cardiac tissue biopsies, proteomic approaches to biomarker discovery in cardiac disease patients have largely focused on detection of circulating ECM secreted constituents or breakdown products [38]. While sampling ECM secreted or breakdown products provides easy detection, this approach is prone to target non-specificity, and additional measures should be taken to establish disease cause and mechanism of action. Proteomic analysis of diseased tissue samples can provide a better insight to changes and distributions of ECM constituents and their functional roles. For example, a proteomics approach was taken to examine ECM changes in human abdominal aortic aneurysms (AAA) samples and successfully identified novel substrate targets of MMP-12 [25]. Briefly, extracts from 0.5 M NaCl and 4 M GuHCl were deglycosylated, separated by 1D electrophoresis and then subjected to in-gel tryptic digest [20]. The digested peptides were then analyzed via LC-MS/MS with a high-mass accuracy analyzer (LTQ Orbitrap XL). Extracts from 0.5 M NaCl and 4 M GuHCl identified a total of 80 and 117 proteins respectively. Normalized spectra counts showed differential clustering of ECM proteins between control and AAA samples highlighting their contrasting ECM compositions. Further in-depth proteolytic analysis was performed on selective ECM remodeling proteins (collagen XII, tenascin, thrombospondin 2, fibronectin) using 1D electrophoresis to detect evidence of their degradation in AAA diseased tissue. Incubation with recombinant MMP-12 resulted in pronounced proteolytic breakdown products of these glycoproteins, and identified them as novel substrates of MMP-12. This study showcased the ECM changes in AAA tissue and established underlying mechanism of action for ECM remodeling pertaining to MMP-12 activity. This proteomic approach can be applied to other tissues or diseased models to probe for associated biomarkers or target substrates with improved selectivity.

3.2 ECM proteomics in disease therapeutic design

The ability of proteomics to simultaneously identify multiple potential biomarkers of disease has outstanding potential for clarifying understanding of disease pathogenesis and identifying novel therapeutic pathways. Categorization of identified proteins into major distinct networks provides a framework for understanding complex proteomic data. This type of system biology approach with large-scale proteomics data can facilitate understanding of molecular mechanism of disease pathology in two ways. First, large-scale proteomic network data can illustrate the crosstalk between signaling protein pathways. Secondly, a system biology approach to understand protein interactions within the ECM may allow identification of novel disease-related pathways by correlating protein partners. A study by Zhang et al. demonstrated that samples from patients with thoracic aortic dissection (TAD) with hypertension vs. healthy tissues yielded differential protein expressions via iTRAQ-labelling and LC-MS/MS analysis [32]. Out of 36 proteins identified that were

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differentially regulated during TAD, several were evaluated and mapped into three major cellular networks involving cell-matrix interaction, ECM remodeling and inflammation response pathways [32]. This study demonstrates the potential for proteomic investigations to not only identify potential biomarker candidates, but also to elucidate potential mechanistic pathways, which represent attractive targets for development of therapeutic interventions.

More recently, the development of ventricular assist devices (VADs) for advanced heart failure has offered a unique opportunity to sample patient's myocardium multiple times and renewed interests in understanding the spatiotemporal changes of ECM and myocytes with this intervention. VADs are surgically implanted pumps that continuously circulate blood from the failing heart to the aorta or pulmonary artery. VADs unload the ventricle, decrease cardiac pressures, and bridge patients to organ transplant or simply provide destination support [39, 40]. Insertion of these devices requires the removal of a core tissue sample for cannulation of the ventricle, providing an opportunity for myocardial tissue sampling under direct visualization. The first sample usually occurs at the time of insertion and a follow up sampling occurs at the time of device removal or cardiac transplantation. Roughly 1.5 to 15% of patients have enough recovery of myocardial function to warrant device removal [41, 42], and re-sampling of an unloaded heart with reverse remodeling is then possible [41]. This tissue sampling offers the opportunity to sample patient's blood and diseased LV tissue simultaneously, which permits the proteomic analysis for biomarker discovery and validation of circulating biomarker levels in relation to tissue pathology. This realization has resulted in heightened interest in ECM constituents and their turnovers as potential target for disease diagnosis, monitoring, prognostication and therapeutic interventions [38, 43].

4. ECM proteomics and cardiac disease

Increasingly, studies have reported that ECM remodeling after a cardiac insult is a major contributing factor to disease recovery or progression. The functional role of ECM remodeling in the cardiovascular system has both acute and chronic impacts on tissue function [44]. Following injury, ECM remodeling processes are initiated to promote healing response for repair or pathological remodeling, which may continue to exert detrimental functional effects even after resolution of the inciting cause. This continuous ECM remodeling is a complex multi-phase process that has yet to be fully defined. While fibroblasts are the primary source for ECM synthesis and breakdown in a healthy myocardium, ECM regulation can be modified by macrophages, lymphocytes and myofibroblasts post-injury to contribute to the complexity of the remodeling process [45]. Additionally, many protease enzymes, namely MMPs and TIMPs, are differentially regulated following I/R injury [46, 47] and have been implicated in eliciting downstream signaling cascade events. In a recent review, Patterson et al. provided a detailed summary of MMPs and TIMPs alterations post-MI [46]. However, the functional impact of such alterations in protease availability and activation on myocardial tissue function and ECM properties remains to be investigated. A proteomics approach offers a powerful methodology for in-depth investigation of the molecular changes of ECM proteins during cardiac disease and can provide critical spatiotemporal information to furthering our understanding of disease initiation and progression.

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MMP-9 has emerged as the most extensively studied member of the MMP family due to its regulatory role in ECM remodeling during ageing or injury-induced in cardiac dysfunction. MMP-9 protein level was found to increase in ageing mice, and its genetic deletion attenuates ageing-induced decline in diastolic function [48]. MMP-9 is also upregulated immediately post-MI [47, 49], and its inhibition has been correlated with reduced adverse LV remodeling [50, 51]. Clinically, elevation in baseline circulating MMP-9 has been reported to be predictive of cardiovascular mortality rates at 4-year follow-up in patients with coronary artery disease [52]. Similarly, patients with diastolic heart failure demonstrated increased myocardial collagen turnover linked with higher plasma MMP-9 concentrations [53]. These examples emphasize the potential utilization of MMP-9 as a novel prognostic biomarker for determining the outcome for patients of various cardiovascular conditions. Proteomics studies of large patient populations are currently still needed. Since MMP family members share high sequence homology, some have cautioned the use of MMP inhibitors in cardiovascular treatment due to their broad, non-specific action with little therapeutic benefit in patients [54]. Indeed, a recent study examining the effect of MMP inhibitor in treating early remodeling post-MI found that LV remodeling index was similar between placebo and treatment patient groups [54]. Moreover, most MMP inhibitors have relatively broad specificity, indicating the critical need to re-evaluate their therapeutic index during drug development [55]. These findings underline the crucial need for more in-depth proteomic analysis of MMP signaling networks and their crosstalk to address the issue of MMP inhibitor specificity. Proteomic analysis has the potential to augment the observed temporal relationship of MMP-9 in progression of coronary artery disease by providing additional data on the protein networks that involve MMP-9 to identify changes in its interacting protein partners (both substrates and inhibitors). Furthermore, an improved understanding of MMP substrates and their roles in tissue remodeling can optimize the target selection for efficient and selective inhibition. Zamilpa et al. performed a proteomic analysis to identify *in vivo* substrates for MMP-9 in LV remodeling post-MI [21]. Comparing post-MI LV extracts from wildtype (WT) and MMP-9 null mice using 2DE, 31 proteins spots were found to be statistically significant between two groups with each spot associated with multiple proteins analyzed by ESI-MS/MS. More importantly, 85 proteins were found to be potential MMP-9 substrates (61% intracellular, 34% extracellular and 5% membrane proteins) [21]. For the first time, several ECM components and their breakdown products were identified using 2DE and demonstrated to be statistically significant different between the WT and MMP-9 null mice. This was the first evaluation of MMP-9 *in vivo* targets established in a clinically relevant MI model using proteomics. A system biology approach using proteomics to study MMP-9 protein interacting partners involved in ECM remodeling can facilitate the development of new therapeutic candidates that are non-MMPs for cardiovascular disease.

4.1 Cardiac ECM secretome

One approach to understanding ECM production in health and disease is to examine the secretome of various cell types in tissue culture. Since the ECM matrix is continually produced as a result of cellular protein production and secretion, secretome analysis offers a platform to investigate ECM production under differing experimental conditions. Proteomics studies aimed at profiling secretory molecules can help dissect the key mediators

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leading to tissue repair vs. maladaptive remodeling. Secretory molecules released from injured tissue during an insult trigger a cascade of downstream signaling events to promote either a regenerative tissue repair response or a pro-inflammatory response forming scar tissue. Following acute MI, surgical revascularization therapies result in re-oxygenation of ischemic myocardium, restoring tissue homeostasis and function. However, even short-term hypoxia results in local ischemic tissue damage and upon reperfusion, this ischemic myocardium undergoes additional stress due to ROS free-radicle production. Li et al. reported a quantitative iTRAQ proteomic study examining the secretome of a cultured cardiomyoblast cell line during hypoxia and re-oxygenation as a model for MI and reperfusion [33]. A total of 860 iTRAQ-labelled proteins were separated using RP column and identified using LC-MS/MS analysis. More importantly, the secreted proteins were classified into several biological processes to identify major signaling pathways involved during hypoxia or reoxygenation. The upregulation and downregulation of these signaling pathways were also evaluated to provide a more comprehensive understanding of the pathways regulated by each of the two distinct stress stimuli individually. The data showed that hypoxia in cardiomyoblast H9C2 line promotes secretion of molecules associated with angiogenesis, inflammation, collagen synthesis and ECM organization. In contrast, reoxygenation suppresses inflammation response, TIMP activity and induces cell adhesion and survival [33]. A key finding of the study points to the reoccurring role of ECM protein dynamic regulation throughout the course of hypoxia and reoxygenation to indicate its fundamental role in myocardial tissue response to I/R injury. The degree of ECM regulation and remodeling is a contributing factor in determining the outcome of tissue repair or maladaptive remodeling to form scars.

Secretome analysis has also been applied to characterize human disease via *in vitro* culture system. Taga et al. analyzed the degree of collagen O-glycosylation in skin fibroblasts from normal and OI patients [34]. The collagens from culture media of these two groups of fibroblasts were purified via hydrazide SPE to enrich for O-glycosylations (galactosyl-hydroxylysine (GHL)/glucosyl-galactosyl-hydroxylysine (GGHL)) and analyzed using LC-MS/MS. To further quantify the relative glycosylations on collagens, cultured fibroblasts were labeled using SILAC. A total of 14 GHL/GGHL sites in collagen I were identified via O-glycopeptides enrichment from both groups. While all the O-glycosylations sites were shared between the two groups, OI collagen showed significant increase in GGHL quantity and not GHL amount [34]. This secretome analysis provides an improved characterization PTMs of collagen I between normal and OI patients.

4.2 Cardiac ECM secretome and cell therapy

Similar proteomic analysis can be applied to compare the secretory molecules released by different cell types to explore options for cell therapy for cardiovascular indications. A proteomics study of ECM secretion by primary murine cardiac fibroblasts (CFs) analyzed the secretome post-transfection with two important miRNAs (miR) centrally involved in fibrosis [56]. The protein samples were separated using 1D electrophoresis and excised bands analyzed by LC-MS/MS analysis. A total of 245 ECM proteins were identified, including low abundant proteases, basement membranes, GFs and cytokines. Critically, this proteomic approach directly examined the network of proteins regulated by miR-29b and

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miR-30c, and validated *in vivo*. This approach highlights the novel application of proteomics in offering previously unknown targets regulated by two key miRNAs involved in cardiac fibrosis. Moreover, proteomic analysis was applied to compare the secretome released between cardiac stem cells (CSCs) and cultured neonatal rat ventricular myocytes (NRVMs) [57]. The samples were separated via RPLC and analyzed using ESI-MS/MS to reveal that 15 out of 83 identified proteins met the paracrine and autocrine factor criteria. In particular, different cytokines specific to either cell type were found to play a role in cell proliferation. Additionally, many secreted ECM proteins whose effect on stem cell proliferation remained to be investigated. For instance, the functional roles of periostin, tropoelastin and collagen VI secreted by NRVMs have not been described. This study is a prime example of how relative secreted protein concentration can interact and crosstalk to impact cellular behavior. When considering cell therapy for regenerative applications, the complexity imparted by multiple cell types and signaling pathways should be considered to optimize the regenerative tissue response.

4.3 ECM proteomics in whole cardiac tissue

In addition to common obstacles facing ECM proteins identification as previously mentioned, their identification can be particularly challenging in a highly cellular and metabolically active tissue, such as myocardium, due to its abundance of highly organized macromolecular sarcomeric proteins and rich mitochondrial content. The relative insolubility of macromolecular sarcomeric components, particularly in the rigor state, poses as an additional hurdle for protein prefractionation and extraction in myocardial tissue. Finally, the heterogeneity of cell types present in myocardial tissue, such as myocytes, fibroblasts, endothelial cells and vascular smooth muscle cells, represent an additional challenge to proteomic analysis, particularly if whole tissue homogenates are used.

Recent advancements in proteomic techniques have enhanced the ability to detect ECM proteins within whole cardiac tissues. This has tremendous impact on capturing the dynamic ECM remodeling to delineate the spatial and temporal information on ECM functional role in disease onset and progression at the tissue level. Specifically, decellularization technique stemmed from tissue engineering has propelled the field of proteomics by allowing for visual confirmation of cellular removal and resultant ECM isolation during initial prefractionation. A seminal paper by Diangelos et al. described the first utilization of three-step protein extraction approach to successfully increase the ECM protein yields in human aorta [20]. Of the 103 ECM proteins identified, roughly a third has never been previously reported in vascular proteomics literature. The sequential three-step extraction method begins with 0.5 M NaCl to extract loosely bound ECM proteins, followed by low concentration of SDS (0.08%) to decellurize the aorta, removing the majority of cellular and nuclear proteins without disrupting ECM proteins. Lastly, samples are subjected to 4 M GuHCl extraction to solubilize ECM components. A critical addition to this method is the deglycosylation of glycosaminoglycan side chains in all three extracts to achieve separation and resolution of glycoproteins and improve the identification of proteoglycans. All three extracts were subjected to 1D electrophoresis prior to analysis by LC-MS/MS. In this study, several novel glycoproteins were discovered for the first time at the protein level in human aorta. Examining the subproteomes of each extraction step reveals different proteases and

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their degradation products, allowing inference into the compartmentalized proteolytic process involved in production of both ECM and non-ECM proteins [20]. The ability of this method to discover new protein targets in human sample and their relevant substrates underlines the immense potential of its application in clinical specimens to investigate tissue dysfunction and associated mechanisms.

The Mayr lab later applied a similar proteomic method to study cardiac ECM remodeling in a porcine model of I/R injury [26], where many scarce ECM proteins were reported for the first time in cardiac tissue. Again, the tissue samples underwent stepwise protein extractions, followed by 1D gel separation prior to LC-MS/MS analysis. Interestingly, the authors found a discordant regulation of mRNA and protein expressions between the focal injury site and the border zone. Although profibrotic genes were increased in both regions, ischemic injury was more pronounced at the focal lesion than the border zone. The system proteomic approach also dissects the temporal signature of early and late stage cardiac remodeling with TGF-β centrally involved at the interaction network [26]. This study demonstrates the ability of powerful proteomics approach on delineating the spatiotemporal aspect of the multi-stage ECM remodeling post-MI, and offering insights to new therapeutic target discovery. Subsequently, several studies have adapted tissue decellularization methods to enhance ECM protein enrichment and extraction in cardiac tissues. A study targeted at cECM compared various strategies to optimize isolation of soluble, cellular and insoluble protein fractions from mouse hearts [18]. Mouse LV tissues were decellularized to remove cellular proteins followed by acid extraction, deglycosylation and the cECM enriched insoluble fraction was solubilized in GuHCl. The proteins were then separated by 1D gel before LC-MS/MS identification. The authors subsequently applied proteomic analysis of LV ECM composition to study the role of secreted protein acidic and rich in cysteine, a collagen-binding protein, in age-related LV stiffness and dysfunction [31]. Mouse LV tissues were homogenized, prepared and analyzed by ESI-MS/MS to identify relative difference in 321 proteins between WT and SPARC-null mice, 44 of which were ECM proteins. These methods demonstrate the discovery and identification of many scarce ECM proteins, enabled by tissue decellularization and resultant ECM protein enrichment (Figure. 1). Importantly, whole tissue ECM proteomics is a powerful tool to analyze the functional roles of ECM remodeling and provides the ability to capture spatial and temporal ECM protein regulations.

4.4 Cardiac ECM proteomics and tissue engineering

Adaptation of decellularization technique has significantly enhanced the efficiency of ECM enrichment for proteomic analysis to study the functional role of ECM remodeling in disease pathogenesis. Continued work is still needed to fully elucidate the ECM remodeling events involved in cardiovascular developmental and regenerative responses. The comprehensive understanding of constructive ECM remodeling has potential to further inform future strategies in engineering biomaterials appropriate for tissue repair and regeneration. For instance, ageing has been shown to compromise normal ECM regulation, thus altering tissue function. Native ECM turnover becomes dysregulated in ageing cardiac tissue, where MMP-9 expression increases with age and contributes to increase fibrotic tissue deposition, increasing passive stiffness with resultant diastolic functional decline [53, 58]. Employing

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ECM proteomics to investigate differences between young vs. aged animal or human cECM can provide developmental insights. This information can be useful to inform biomimetic approach in engineering the biologically relevant tissue. That is, clinical application of engineered cardiac tissue in a young patient may have different requirements than those needed for an older patient.

Although the ECM has been implicated in many cellular behaviors, the molecular mechanisms of how reciprocal interactions between cell-matrix alter cellular response remain unclear. Emerging areas of interests therefore involve deciphering the role of ECM in stem cell differentiation and regenerative application. Recent studies have reported that ECM produced by mesenchymal stem cell (MSCs) derived from young donors exhibited higher protein expressions than those derived from aged donors [59]. Interestingly, senescent fibroblasts can be restored to a more youthful phenotype when repopulated onto juvenile ECM [60], indicating the biological influence of ECM age on cellular response. These findings can inspire future biomimetic tissue engineering strategies, allowing for efforts to be focused on critical tissue specific cellular functions to enhance neotissue formation. These examples indicate that ECM biology, development and remodeling have major implications in stem cell therapy and engineering biologically functional constructs for regenerative applications.

5. Concluding remarks

The ECM constitutes a dynamic modulatory component to tissue function and homeostasis beyond its basic structural and mechanical properties. Years of research have substantiated the functional impact of ECM synthesis in normal tissue development and ECM remodeling in both health and disease. However, the molecular pathogenesis of various spatiotemporal aspects of ECM remodeling in diseased tissue remains lacking. Proteomic approaches offer potential to probe the dynamic events occurring within the ECM and further our understanding of tissue development, turnover and disease. ECM enrichment via protein fractionation or decellularization of whole tissue significantly enhances the identification of many scarce ECM proteins. Proteomics profiling of ECM secretome provides another mean to quantify the ECM components associated with cell type of interest. With large-scale ECM proteomics information, future studies will require bioinformatics tool to facilitate a system biology approach to analysis, quantification and clustering of protein data based on their functions and interactions. Recent advents in cardiac ECM proteomics have potential clinical applications with identified ECM molecules and breakdown products showing promise in biomarker discovery, prognostic monitoring and therapeutic development. Future work is needed to explore these possibilities in cardiovascular disease and intervention.

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Abbreviations

CSC	cardiac stem cell
ECM	extracellular matrix
FGF	fibroblast growth factor
ICM	ischemic cardiomyopathy
I/R	ischemia/reperfusion
LV	left ventricle
MI	myocardial infarct
MMP	metalloproteinase
NRVM	neonatal rat ventricular myocyte
PTM	post-translational modification
SDS	sodium dodecyl sulfate
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of metalloproteinase
VEGF	vascular endothelial growth factor
VAD	ventricular assist device

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Cardiac extracellular matrix proteomics: Challenges, techniques, and clinical implications

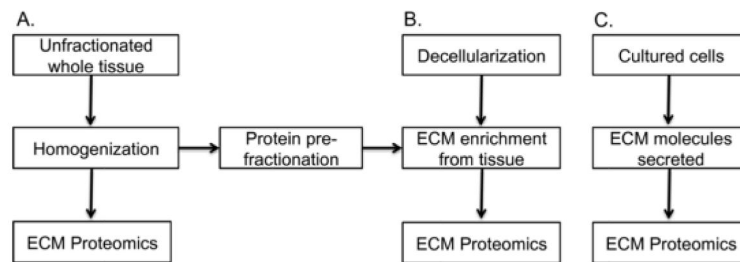

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Figure 1. Diagram depicting three major approaches currently utilized in ECM proteomics
Methods to investigate ECM proteomics via (A) homogenization of whole tissue, (B) ECM enrichment via protein prefractionation or tissue decellularization, and (C) ECM molecules secreted from cultured cells.

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Table 1

Summary of Sample Prefractionation, Separation and ECM MS Methods

This table summarizes the lists of cardiac ECM proteomics approaches utilized in the literature. GHL, galactosyl-hydroxylysine; GGHL, glucosyl-galactosyl-hydroxylysine.

Authors & Year	Sample Preparation	Separation/Labeling Techniques	MS Method & Equipment	Proteins ID's	ECM ID's
Abonnenc et al. 2013, [56]	Secretome	1D gel	LC-MS/MS (LTQ-Orbitrap XL)	N/A	245
Barallobre-Barreiro et al. 2012, [26]	decellularization (0.5M NaCl, 0.08% SDS, 4M Guanidine-HCl)	1D gel band excision followed by HPLC	LC-MS/MS (LTQ-Orbitrap XL)	N/A	> 100
de Castro Brás et al. 2013, [18]	LV Decellularization followed by homogenization	1D gel band excision followed by HPLC	LC-MS/MS (LTQ Orbitrap Velos)	N/A	N/A
de Castro Brás et al. 2014, [31]	LV Decellularization followed by homogenization	1D gel band excision followed by HPLC	ESI-MS/MS (LTQ Orbitrap Velos)	321	44
Didangelos et al. 2010, [20]	Prefractionation by decellularization (0.5M NaCl, 0.08% SDS, 4M Guanidine-HCl)	1D gel band excision followed by HPLC	LC-MS/MS (LTQ-Orbitrap XL)	N/A	105
Didangelos et al. 2011, [25]	Prefractionation by decellularization (0.5M NaCl, 0.08% SDS, 4M Guanidine-HCl)	1D gel band excision followed by HPLC	LC-MS/MS (LTQ-Orbitrap XL)	197	53
Li et al. 2014, [33]	Secretome	iTRAQ RP C_{18} column	LC-MS/MS (QStar Elite)	860	N/A
Lundby et al. 2013, [19]	Whole tissue homogenization, titanium oxide enrichment for phosphopeptides	RP C_{18} column	LC-MS/MS (LTQ Orbitrap Velos)	4246 (Phosphoproteins)	N/A
Stastna et al. 2010, [57]	Secretome	RP C_{18} column	LC-MS/MS (LTQ ion trap or LTQ Orbitrap)	83	N/A
Taga et al. 2013, [34]	Hydrazide for O-glycopepetides	SILAC RP C_{18} column	LC-MS (3200 QTRAP)	14 GHL/GGHL sites	N/A
Tian et al. 2014, [27]	LV tissue homogenization, hydrazide SPE for N-glycopeptides	RP C_{18} column	LC-MS/MS (Q Extractive)	1352 sites, 694 proteins (Glycoproteins)	N/A

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Authors & Year	Sample Preparation	Separation/Labeling Techniques	MS Method & Equipment	Proteins ID's	ECM ID's
Zamilpa et al. 2010, [21]	LV homogenization	2DE	ESI-MS/MS (LTQ linear ion trap)	85 potential MMP-9 substrate proteins	29
Zhang et al. 2015, [32]	Secretome	iTRAQ	LC-MS/MS (TripleTOF 5600)	36	N/A