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Cardiac Extracellular Matrix Modification as a Therapeutic Approach

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Abstract

The cardiac extracellular matrix (cECM) is comprised of proteins and polysaccharides secreted by cardiac cell types, which provide structural and biochemical support to cardiovascular tissue. The roles of cECM proteins and the associated family of cell surface receptor, integrins, have been explored in vivo via the generation of knockout experimental animal models. However, the complexity of tissues makes it difficult to isolate the effects of individual cECM proteins on a particular cell process or disease state. The desire to further dissect the role of cECM has led to the development of a variety of in vitro model systems, which are now being used not only for basic studies but also for testing drug efficacy and toxicity and for generating therapeutic scaffolds. These systems began with 2D coatings of cECM derived from tissue and have developed to include recombinant ECM proteins, ECM fragments, and ECM mimics. Most recently 3D model systems have emerged, made possible by several developing technologies including, and most notably, 3D bioprinting. This chapter will attempt to track the evolution of our understanding of the relationship between cECM and cell behavior from in vivo model to in vitro control systems. We end the chapter with a summary of how basic studies such as these have informed the use of cECM as a direct therapy.

Keywords

ECM mimetic; Recombinant ECM; Cardiac therapy; Cardiac repair; Bioink; Bioprinting

1 In Vivo Models to Evaluate Macroscale Function of cECM

Collagen type I (Col I) is a fibril-forming collagen and the most abundant component of cECM with well-described composition-function relationships. Col I is secreted from cells

as procollagen, which contains a triple-helical domain flanked by non-collagenous propeptides. When the propeptides are removed, the triple-helical domain can self-assemble into fibrils which are further stabilized by lysyl oxidase-mediated covalent cross-links between and within the triple helix [1]. The structure of Col I is the primary means by which strength and stiffness are conferred to the heart and vascular tissues. If one tracks the density and organization of Col I in the developing mouse embryo, it increases over time and reaches a peak at day 12.5 in all layers of heart tissue (i.e., epicardium, myocardium, and endocardium) [2]. The amount of Col I relative to other proteins then drops, allowing the heart to become more compliant and thus better able to respond to the contractile apparatus of maturing cardiomyocytes of cardiac muscle [3]. Col I is also important for repair of heart tissue following disease or injury especially when such a state is accompanied by loss of cardiomyocytes. Cardiac fibroblasts are recruited to compensate for lost muscle, and they do so primarily by secreting large amounts of Col I. It is not surprising therefore that mutations in the $\alpha 2(I)$ chain impair cardiac development [4] and also restrict cardiac repair following myocardial infarction [5]. In the vasculature, changes in Col I synthesis can cause vascular abnormalities leading to aneurysms and cerebral artery dissections in patients with abnormal procollagen genes and to varicose veins where Col I synthesis is upregulated [6, 7].

Collagen type III (Col III) is another fibrillar collagen of cECM which is prevalent in tissues which require compliance [8]. Col III forms a more compliant network composed of finer fibers than fibers formed from Col I [9]. Col III is necessary for normal cardiovascular development, as it colocalizes with Col I and regulates Col I fibrillogenesis [10]. Knockout mice have shortened lifespans due to rupture of major blood vessels and abnormal formation of Col I fibrils in the blood vessels and heart [11]. Mutations on Col III have also been linked to the vascular phenotype of Ehlers-Danlos syndrome which causes spontaneous arterial dissections, primarily of medium-sized vessels [12]. The ratio of Col I to Col III is often referenced as a means to gauge the relative stiffness of cardiovascular tissue with age or disease. The ratio of Col I to Col III is high in comparably stiff neonatal hearts. The ratio then decreases for some time after birth until stabilization in adulthood, contributing to the relative compliance of the adult heart [3]. This ratio is also affected by blood volume overload as seen during pregnancy where the Col I/Col III ratio decreases to accommodate the movement of increased blood volume. The decreased Col I/Col III ratio with pregnancy is reversible postpartum [13]. The reversibility of this change indicates that ECM homeostasis in the heart is active throughout the lifespan and that a healthy heart can repeatedly remodel to accommodate major changes in cardiovascular load.

Col IV is the most abundant component of arterial basement membranes of cECM [14] and plays a role in both cardiac tissue stabilization and angiogenesis. In contrast to Col I and Col III, Col IV is a non-fibrillar collagen composed of six distinct α -chains which assemble to form three heterotrimers $\alpha 1\alpha 1\alpha 2$, $\alpha 3\alpha 4\alpha 5$, and $\alpha 5\alpha 5\alpha 6$ [15]. The $\alpha 1\alpha 1\alpha 2$ and $\alpha 3\alpha 4\alpha 5$ heterotrimers are essential to cardiac development and angiogenesis and dominate in vascular basement membranes. However, Col IV is not essential to the deposition of proteins in the basement membrane, only to its integrity. Col IV knockout mice develop until embryonic day 9.5, and basement membrane proteins deposit appropriately in the developing embryos, and the embryos showed normal organ development and beating hearts. However, the knockout is lethal in the following days due to structural defects of the

basement membrane, primarily in the vasculature. At embryonic days 10.5–11.5, the Col IV-deficient mice developed pericardial bleeding and dilated blood vessels, indicating that although development of the major organs and vasculature appeared normal at early stages, subtle, likely subcellular differences in the organization of the basement membrane are eventually lethal [16].

Fibronectin is a glycoprotein involved in the regulation of cell adhesion and which provides tissue mechanical properties by binding of cells to cECM components. It is composed of two subunits connected by disulfide bonds. The subunits contain repeating modules I, II, and III which comprise the functional domains of the protein [17]. Fibronectin is essential for development and repair of cardiac tissue. Mice that lack the fibronectin gene die in early embryonic development and exhibit a variety of defects of cardiac and vascular tissues [18]. *Mice that lack fibronectin also respond less favorably to cardiac injury due, at least in part, to decreased proliferation and survival of cardiac progenitor cells relative to wild type* [19]. *Cardiac progenitors that do survive in this model tend to localize to fibronectin synthesized prior to induction of the knockout* [19]. In vivo studies have also led to an understanding of fibronectin interactions with integrins. Knockouts of the main fibronectin receptor, integrin $\alpha 5\beta 1$, led to defects similar to those of fibronectin knockouts, whereas other integrins which can bind fibronectin, $\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 8\beta 1$, $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 6$, and $\alpha IIb\beta 3$, lead to less severe defects, which indicates independent functions of the fibronectin receptor integrins during development as well as functional redundancy between fibronectin receptors other than $\alpha 5\beta 1$ integrin [20]. While global knockdown of the $\alpha 5$ integrin subunit was embryonic lethal and showed severe cardiovascular defects, it was difficult to discern the exact implications for specific cell types. *Chen et al. [21] addressed this difficulty by generating conditional fibronectin knockouts specific to endothelial, smooth muscle, and pharyngeal mesodermal cells. Interestingly, they did not observe defects in vascular morphogenesis, suggesting that either there is redundancy of production of fibronectin by the cardiovascular cell types tested here or that an alternative cell type is a potent synthesizer of fibronectin. Indeed, cardiac fibroblasts are a major producer of fibronectin in the heart [22] and a likely source of the observed production redundancy of fibronectin in cardiac tissues. Further, the study by Chen et al. [21] did not examine the effects of conditional knockouts on the development of cardiac muscle, though the results in the vasculature suggest multiple cell types are capable of contributing fibronectin to the cECM to thereby to support cardiac muscle.*

Elastin is the major component in elastic fibers of cECM, which provide elastic recoil to tissues subject to repetitive stress. It is assembled from its soluble, monomeric form, tropoelastin, which consists of alternating hydrophobic and hydrophilic regions. Elastin has been shown to be essential to proper development of the heart and vasculature [23]. Mice lacking the elastin gene die a few days after birth due to aortic stenosis, suggesting elastin in the heart is important not only for chamber and vessel recoil but also for regulating arterial smooth muscle growth. Artery sections from patients with supravalvular aortic stenosis caused by mutations in the elastin gene show an increased number of lamellar units and significant fibrosis, indicating that the role of elastin includes regulation of ECM synthesis in vessel lamellae [24]. Levels of elastin in the developing mouse heart are low early in development and peak at embryonic day 16.5 in the myocardium and endocardium, while

elastin levels peak later in the epicardium [2]. The dynamic nature of elastin synthesis with development is likely essential to the changing elasticity of cardiac tissue, which changes to accommodate additional stresses as the heart grows and develops. Heterozygous mice which are deficient in elastin exhibit altered arterial morphology and cardiac hypertrophy [25]. Further studies in these heterozygous mice have shown that cardiovascular developmental defects begin to appear at embryonic day 18. The late appearance of cardiovascular defects in these heterozygous mice indicates that elastin is not essential until late in embryonic development, likely due to an increase in cardiac stresses near birth [23].

Laminins are a family of proteins, which are found in the basement membranes of vasculature and cardiac tissue. They are composed of one α , one β , and one γ chain that form a T-shaped heterotrimer. Laminins are named based on the subunits they contain. For example, laminin 511 contains the $\alpha 5$, $\beta 1$, and $\gamma 1$ subunits. These subunits share a common domain structure which contains rod-like domains but differ in their globular domains [26]. The laminins most commonly associated with cardiac and vascular tissue are 111, 411, and 511. Deletions of the $\alpha 1$ chain present in Laminin 111 are lethal at early embryonic stages, which have made discerning its exact functions in development difficult [27]. Laminins 411 and 511 are predominant in vascular basement membranes. Laminin 411 expression is essential to microvessel maturation and affects deposition of other basement membrane components, including Col IV [28]. Laminin 511 expression in vascular basement membranes begins later, about 3–4 weeks after birth, and likely has a role in vessel maturation [29].

In vivo experiments, primarily via the generation of transgenic mice, have established the critical utility of cECM proteins during development and with disease and their significance for the structural integrity of the heart and vasculature. In particular, it is clear that each of the primary ECM proteins of the cardiovascular system is unique in its function in terms of tissue type and developmental time point. Conditional knockout experiments have added to that with information to further delineate cell types involved in matrix synthesis and have revealed details about the roles of ECM in cardiac development even when global mutations are lethal early in the embryonic period. These studies have led to a greater understanding of the major players in the cECM and have established motive for the use of cECM proteins as therapeutics for cardiovascular disease. However, in vivo studies face the unavoidable limitation that one must study the whole organism, which precludes isolation of the effects of a given ECM component on one organ, cell type, or process which is essential to understanding the mechanisms by which cECM affects cell behavior during development, health, disease, and therapy. Understanding these mechanisms is necessary to the development of more advanced cECM-based therapeutics for cardiac diseases and for improving patient outcomes. This has led to the advent of in vitro systems to study the cECM. The central advantage of in vitro studies is the ability to better distill the interplay of specific cell types with particular matrices to discern signaling pathways elicited and functional behaviors attained. In vitro studies allow uncoupling of competing or compensatory factors, which can mask critical interactions missed in in vivo studies.

2 In Vitro Control Systems to Evaluate Cell-cECM Interplay at the Microscale

First, efforts to break down the interplay between specific ECM proteins and individual cardiac cell types involved electrostatic adsorption of ECM proteins to cell culture plates. Plates coated with individual cECM were used to determine the attachment of adult and neonatal cardiomyocytes to several ECM proteins. Adult cardiomyocytes attached most efficiently to laminin and Col IV and bound weakly to fibronectin. Neonatal cardiomyocytes attached well to Cols I, II, III, IV, and V as well as to fibronectin and laminin [30]. The difference in adhesion with age is thought to reflect developmental regulation of extracellular matrix binding via changes in the affinity of cell surface receptor integrins for the ECM and through changes in the expression of Col I receptors during development. Fibronectin, laminin 111, and Col I were compared as substrates for the proliferation, attachment, and differentiation of late endothelial progenitor cells. They were found to attach more strongly to fibronectin and Col I than to laminin and had higher proliferation rates on these surfaces. However, significant differences in endothelial differentiation of the cells on different surfaces were not observed, indicating that either these ECM proteins are not essential for differentiation or that ECM is more important for differentiation that precedes the endothelial progenitor state or that multiple ECM-based signals are required in concert [31]. These simplistic studies of single ECM components led to the study of combinations of cECM proteins in 2D in order to discern how interactions between proteins influence cardiac cell behavior. When human pluripotent stem cells were seeded on combinatorial ECMs composed of Col IV, Lam 111, and heparan sulfate or Col IV, gelatin, and heparan sulfate, a significant increase in CD31 expression was observed over single-factor ECMs [32]. The study implies that cell behavior is guided through a combination of signals from the ECM, several of which are required in order to enhance differentiation. Even greater complexity can be achieved in 2D tissue culture format via the use of cardiac tissue-derived ECM. A biomaterial sheet prepared from decellularized rat hearts spurred enhanced cardiac gene expression when compared to cells cultured without the ECM sheets. However, because the biomaterial was not compared to single ECM proteins, it is unclear whether the added complexity significantly affects cell behavior. The cardiomyocytes also had higher rates of proliferation and viability on the naturally derived cECM sheets compared to cells cultured without ECM [33]. Though intriguing, these types of studies need to be coupled to additional experiments designed to determine which element(s) of the ECM are most critical to trigger a desired cellular effect.

As a whole, 2D experiments have provided beneficial insight into the appropriate culture conditions for cardiac cell types. However, a 2D environment is not physiologically relevant, and in recent years, accumulating evidence has shown that three-dimensional engagement of ECM in vitro, akin to that experienced in vivo, gives rise to augmented and sometimes different cellular behaviors than in 2D [34–36]. Thus, to better recapitulate ECM exposure in tissues, both single and combinatorial protein approaches have been applied in three dimensions. A polymer platform, wherein 3D poly(ethylene glycol) hydrogels cross-linked via native chemical ligation, was used to entrap individual ECM and known combinations of ECM with induced pluripotent stem cells. The hydrogels formed were compatible with

multiwell plate formats, and so the number of conditions (i.e., different combinations and concentrations of ECM) could be scaled. Then by applying a “design of experiments” statistical approach to the platform, systematic optimization of the ECM ratios led to the identification of a formulation of cECM proteins optimized for cardiomyocyte differentiation. The theoretical formulation was confirmed experimentally via immunophenotyping and functional analyses [37]. Natural multicomponent ECMs derived from tissues have also been used in 3D to influence cell differentiation toward cardiomyocytes. Cardiogel, which is derived from cardiac fibroblasts, has been shown to control differentiation of bone marrow-derived stem cells toward a cardiomyocyte phenotype [38]. The components of cardiogel have been characterized and contain laminins, fibronectin, Col I, Col III, and a variety of proteoglycans [39]. However, while multicomponent ECM extracted from tissue can effectively control cell behavior, it is important to note that the composition of these materials is not well defined and batch-to-batch variability can be difficult to resolve. Thus, there are plenty of advantages of using natural ECM extracted from tissue to generate 2D and 3D in vitro models of cardiac tissue, and these include (1) the inclusion of complex biologic functionalities, even when those functionalities are not fully understood, and (2) evolutionarily guided periodicity and coordinated interplay of multiple ECM domains and multiple cellular domains, with (3) resultant sophisticated intracellular signaling that guides remodeling of the ECM and associated cell behavior. However, it can be difficult to study natural ECM proteins because they are difficult to reliably source and they do not all form hydrogels spontaneously in 3D necessitating inclusion of gelation platforms.

Advances of the last several decades have led to the identification of some of the critical motifs of cECM that affect cell behavior. For example, studies utilizing recombinant ECM have shown that the arginine-glycine-aspartic acid (RGD) binding domain on fibronectin is highly sensitive to the surrounding synergy residues and that the GFOGER and GLOGER sites on fibrillar collagens are selective for the $\alpha 2\beta 1$ and $\alpha 1\beta 1$ integrins, respectively [40, 41]. With this improved understanding of ECM activity in mind, we are now moving toward the development of artificial ECM that affects cell behavior. These artificial ECMs typically fall into three categories: “blank” polymer backbones with added functionality, ECM mimetics, and modified natural proteins.

Synthetic polymers, often referred to as “blank slates,” can be modified to include ECM-like domains which add functionality such as cell-binding motifs and matrix metalloproteinase-binding motifs. One commonly used synthetic polymer is poly (ethylene glycol) (PEG). PEG hydrogels functionalized with RGD have been used to direct differentiation of embryoid bodies toward endothelial cells and cardiomyocytes. The addition of the RGD peptides to the PEG hydrogels decreased cell aggregation in the gels and also drove the cells toward endothelium. A reduction in RGD led to an increase in both cell aggregation and cardiomyocyte differentiation [42]. A polyurethane elastomer functionalized with RGD, another cell binding domain YIGSR, and heparin has been shown to reduce platelet adhesion and enhance attachment of endothelial cells to the surface [43]. An E8 fragment of laminin 411 which represents the C-terminal region of the protein has also been shown to increase differentiation of human iPSCs toward endothelial cells and could be combined with a blank slate to study endothelial differentiation [44]. The fragment improves endothelial

differentiation over the entire laminin 411 protein, and endothelial cells created this way showed higher expression of genes associated with vascular development and angiogenesis. Studies using blank slates with added functionality allow for the isolation of the effects of specific domains within either a 2D or 3D system, which addresses several of the problems with using whole, natural ECM proteins by creating platforms which allow for gelation to form 3D structures containing well-defined protein domains. However, creation of these systems requires an understanding of which protein domains influence cell behavior in the desired way and can be limited in the inclusion of synergy sites and lack often necessary secondary, tertiary, and quaternary protein structures.

In an attempt to include synergy sequences or molecular structures of cECM, other groups have attempted to mimic a larger portion of or the entire protein structure with a peptide-based self-assembly approach. *These polymers are intended to recreate the structural, and sometimes biochemical, complexity of ECM and are often termed “ECM mimetics.”* An elastin-like polypeptide hydrogel was designed by combining RGD domains with structural, elastin-like domains that contain repeating hydrophobic residues. A QK peptide that mimics the activity of vascular endothelial growth factor (VEGF) was grafted to the elastin-like polypeptide to enhance endothelial cell proliferation. The material also showed outgrowth of the endothelial cells from their original colonies, indicating the cells are able to remodel the material to begin forming 3D structures [45]. Heparin mimetics have also been used to include VEGF binding ability reflective of the functionality of heparan sulfate in the basement membrane [46, 47]. An electrospun poly(ϵ -caprolactone) material with fiber diameters and arrangement designed to mimic the organization of healthy cECM was found to promote arrangement of cardiomyocytes and formation of organized actin/myosin bands compared to fibers arranged to mimic the ECM of a failing heart [48]. A Col I mimic which forms fibril-like structures and ultimately a hydrogel have also been created; however, to this point, the mimetic lacks cell-binding motifs and therefore is unlikely to be of much therapeutic benefit beyond a structural Band-Aid [49]. ECM mimetics provide well-defined materials with specific properties and functionalities. However, the majority of cECM mimetics replicate either specific functional domains (heparin-mimetic polymers) or the microscale structure and arrangement of the cECM (electrospun poly(ϵ -caprolactone) and collagen mimetic). The elastin-like polypeptide goes one step further by replicating the structure of elastin and including functional domains. However, the domains included are nonnative to elastin. The creation of more complex ECM mimetics which incorporate native functional domains, with microscale structural motifs and associated mechanical attributes that match that of the native protein complex, is essential to the use of ECM mimetics to study ECM interactions and for moving these materials toward clinical therapies.

To this point we have discussed the impact of purified cECM components or synthetic mimics of cECM, but it can also be useful from a basic science and applications standpoint to couple cECM with synthetic modifications. cECM can be altered using synthetic components to alter cell or growth factor binding or change mechanical properties of a scaffold or tissue. Col I can be modified to add nonnative cysteines, which can interact with a PEG cross-linker to immobilize growth factors. When the growth factor TGF- β 1 was immobilized within a Col I gel in this manner, the gel induced myofibroblast differentiation [50]. The growth factor immobilization is a synthetic way to replicate the natural growth

factor binding of the ECM. These modifications can allow a single component system to harbor multiple functionalities of the ECM. Synthetic modifications to cECM can also be made to tune mechanical properties. A cell-derived ECM has been cross-linked with tunable density using the naturally derived cross-linker, genipin. The material could be tuned to have an elastic modulus between approximately 1 and 10 kPa, which brings the stiffness of the material into the range for the neonatal rat heart (4–11.4 kPa). This cross-linked ECM substrate was supportive of cardiomyocyte differentiation compared to the uncross-linked ECM [51]. The ability to tune the mechanical properties of ECM with synthetic modifications addresses one of the major concerns for the use of natural ECM to study cell-ECM interactions because these modifications could allow creation of 3D structures with appropriate mechanical properties from ECM which does not form a hydrogel on its own. This can aid in decoupling of mechanical stiffness from ECM interactions in ECM studies and provide a platform for the study of a larger number of natural ECM components.

The culmination of the basic in vitro studies described above lays the groundwork for developing ECM-based biomaterials for cardiac tissue engineering applications on the road to therapeutic tissue replacement. The earliest studies using ECM for cardiac regeneration in vivo involved the injection of ECM at the site of injury. Col I injected at the site of myocardial infarction has been shown to improve ejection fraction following treatment and to reduce scar tissue formation when compared to control animals [52]. A bone marrow-derived ECM has also been injected into the heart following myocardial infarction. The rationale for the use of bone marrow-derived ECM was that hematopoietic-derived progenitor cells contribute to angiogenesis following injury. The injection led to decreased apoptosis and lower macrophage counts at the infarct border after 7 days. A decrease in the fibrotic area and increased angiogenesis were also observed [53]. Additionally, several studies have used cECM as an injectable therapy following myocardial infarction. An injectable porcine pericardial matrix gel used as a therapeutic was analyzed by mass spectroscopy to determine the major matrix components in the material. The material contained a large number of ECM components, including Col I, Col IV, and elastin, as well as a variety of other proteins, primarily collagens. Interestingly, the matrix did not contain laminin or Col III, both of which are important components of the cECM. The material increased formation of vasculature and caused infiltration of c-kit⁺ stem cells into the infarct region [54]. Intramyocardial injection of decellularized cardiac ECM has also been shown to increase left ventricular wall thickness and improve heart function following myocardial infarction [55]. Injection of ECM for cardiac repair following myocardial infarction has shown the power of the ECM in cardiac regeneration. However, injection of ECM does not allow for the creation of organized ECM structures, and these therapies did not demonstrate recovery of cardiac muscle mass, which will be essential for full recovery following cardiac muscle damage.

One potential means to recover cardiac muscle mass is to incorporate cells into materials used for cardiac repair as a means to engineer new tissue. A decellularized cardiac ECM has been used as a scaffold for cardiac tissue repair. The scaffold was seeded with cardiomyocytes, fibroblasts, and mesenchymal stem cells in separate studies with good cell viability. Cells also showed elongated morphologies, and scaffolds seeded with cardiomyocytes began to beat synchronously after a few days of culture on the scaffold [56].

There can also be difficulties using only native ECM as a scaffold for tissue repair because natural cECM forms a soft hydrogel (storage modulus around 100 Pa) in comparison to native myocardium [57]. *This mismatch in mechanical properties can mean cells seeded in the material are receiving mixed signals from the ECM and material mechanics, which can alter cell behavior. The softness of the gel can also make it difficult to secure to the epicardial surface, and it is likely to break down under the stresses of cardiac muscle contraction or blood flow. The native ECM has also been combined with fibrin to enhance stiffness and associated manipulation of the scaffold for therapy. The resulting gel was shown to have mechanical stiffness spanning the range of native myocardium (32–46 kPa) with the capacity to promote differentiation of cardiac progenitor cells isolated from pediatric patients with heart defects toward cardiomyocytes* [58, 59].

Organization of cardiomyocytes prior to therapeutic transplantation might also be facilitated by cECM. One method, which has been used to attain this goal, is 3D bioprinting. Bioprinting allows for the controlled deposition of materials and has the potential to create more organized ECM-based structures. A decellularized ECM bioink has been used to print a cell-laden structure which supports cell viability and improves cardiac differentiation over a Col I control scaffold using an extrusion printer [60]. The viscosity and associated printability of decellularized cECM have been improved through vitamin B12-based photocross-linking. This approach allowed for the decellularized ECM to be printed with high fidelity using an extrusion bioprinter and for the final mechanical stiffness of the construct to match those of native myocardium. The final construct supported cardiac progenitor cell proliferation and improved cardiomyocyte differentiation [61]. However, most extrusion printers are not capable of creating structures with features at a scale that can be discriminated by cells (i.e., 1–100 microns). A multiphoton-based 3D printing system has been developed to print an ECM-based tissue patch for cardiac regeneration with submicron patterning. The scaffold was then seeded with cardiomyocytes, endothelial cells, and smooth muscle cells in a 2:1:1 ratio. The submicron scale patterning of the patch replicated the pattern of fibronectin expression in the native heart and created channels into which the cells quickly settled, providing organization for the tissue and smooth propagation of electromechanical signals across the patch. The patches were implanted in mice following myocardial infarction, and the treated mice showed improved cardiac function, smaller infarct size, and vascularization of the infarct region [62]. These bioprinted structures for myocardial repair show the potential of the method to create structures with improved organization of ECM-based materials and to improve outcomes following myocardial infarction. However, the majority of these structures are thin patches, which limit the efficacy of the structures in promoting full thickness repair of tissue.

Vascularization of tissue grafts for myocardial repair is a major challenge for moving from the creation of thin patches to larger structures. For the creation of large vascularized structures, acellular vessels have been used to create vascular grafts. These include a decellularized fibrin graft which has been implanted into sheep and showed graft endothelialization [63] and a decellularized vessel created by culturing human vascular smooth muscle cells on a biodegradable polymer which has reached clinical trials [64]. However, ECM-based vascular grafts continue to face challenges, particularly balancing the need for cross-linking with calcification in vivo. This calcification occurs partially due to the

use of chemical cross-linkers like glutaraldehyde, which can have toxic effects. However, the use of natural cross-linking agents including genipin and procyanides has been shown to inhibit calcification of vascular grafts [65]. The creation of larger-scale vascular grafts using ECM has proven the potential of ECM as a material for the creation of vascular channels. However, it is important to note that the creation of thicker engineered cardiac tissues will not only require creation of larger channels for perfusion but also the creation of complex vascular networks. This subject is under development by several groups globally and has been well-reviewed [66–68]. Successful creation of vascularized thick tissues will promote the development of even larger cardiac tissue structures, perhaps even a cardiac graft in the distant future. Though far off, efforts have already been initiated to use 3D printing to create complex biological tissues including cardiac tissues. An embryonic chick heart has been printed using a soft bioink (alginate) with the support of a gelatin slurry. The structure showed open internal chambers with complex architecture, an advantage of this printing method [69]. However, while the material used to print this structure is a soft material with a modulus of <100 kPa, the material is not able to support cell growth or differentiation and cannot be remodeled by cells. So, while the ability to print soft materials in complex structures is a major advance toward printing viable cardiac tissues, modifications will be required in order to incorporate cells into these complex structures.

3 Concluding Remarks

The essential role of cECM in supporting tissue structure and dictating cardiac cell behavior creates opportunities for a wide range of engineered ECMs, which might ultimately be used to therapeutic effect. Indeed the development of cECM-based clinical therapeutics for the treatment of cardiac and vascular disease is well underway. Proposed therapeutics range in complexity from cell-free scaffolds to thick, vascularized structures. Whether and to which extent these approaches will see significant clinical impact is yet unknown. There are many critical factors that, if not addressed, will limit clinical success, and most of these – including batch variation of natural cECM, compliance mismatch of engineered cECM with cardiovascular tissue, cellular disorganization in engineered cECM, and poor vascularization of thicker cECM-based tissues – have been addressed in this review. Many of these critical factors might be surmounted with a better understanding of the signaling pathways that link integrin engagement of cECM to effector outcomes. We know that focal adhesions or costameres (in the case of cardiomyocytes) are formed with integrin engagement and that these are primarily linked to functions of the cytoskeleton. But the mechanisms by which engagement of different integrins yields varied behavioral outcomes linked to the action of the cytoskeleton (e.g., adhesion, proliferation, migration) are underexplored. Also underexplored are cECM-triggered signaling pathways that may begin with integrin engagement but bypass cytoskeletal components; chief among these is stem cell differentiation.

There is now clear evidence from our group and the work of others that stem cell maturation and differentiation and their respective normal function are critically dependent on the temporal and spatial specification of cECM during developmental [70] and with ex vivo stem cell culture [71–77]. As one of many examples, mesoderm specification has been linked to $\alpha 5\beta 1$ integrin activation. Engagement of this integrin by ECM (especially laminin

511/laminin 111 and fibronectin) modulates BMP4 expression, which together with Wnt, fibroblast growth factor, and transforming growth factor- β /nodal/activin signaling mediates this differentiation [77]. It has also been shown that peptide activation of this integrin can drive osteogenic differentiation of mesenchymal stem cells via the Wnt/ β -catenin pathway activated via PI3K/Akt signaling [78]. In addition, engagement of fibroblast-derived ECM via β 1, α 2, and α 3 integrins in human embryonic stem cells has been shown to activate the Wnt/ β -catenin pathway via the MEK-ERK pathway, which drives endoderm differentiation [79]. Finally, fibronectin/integrin β 1/ β -catenin signaling was shown to promote the emergence of mesoderm from induced pluripotent stem cells. This study was the first to establish a direct link between elements of the focal adhesion, namely, integrin-linked kinase (ILK), with GSK3 β [80], the primary antagonist of β -catenin, and all of this seemingly untethered to cytoskeletal dynamics. We predict that cECM-based therapeutics that tap our growing understanding of cECM-linked signaling pathways will better control cell behavioral outcomes and lead to significant clinical benefit (Figs. 1, 2, and 3).

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Fig. 1.

Timeline showing the lethal age of cECM and integrin mutations. Laminin $\alpha 1^{-/-}$ is lethal at embryonic day 7.5, and the embryo is resorbed due to defects of the basement membrane [81]. $\alpha 5\beta 1$ integrin $^{-/-}$ is lethal at embryonic day 8, and embryos show a variety of cardiac and vascular defects. The image compares a wild-type embryo to a knockout embryo at embryonic day 8 [20]. Fibronectin $^{-/-}$ shows similar defects to the $\alpha 5\beta 1$ integrin $^{-/-}$ and is lethal at embryonic day 8.5 [18]. Col IV $^{-/-}$ is lethal at embryonic day 10.5–11.5 due to pericardial bleeding (shown in the image) and rupture of major blood vessels [16]. Elastin $^{-/-}$ is lethal in the neonatal period due to aortic stenosis, which is shown in the image [24]. Col III $^{-/-}$ leads to abnormal cardiac development and the rupture of major blood vessels after birth [11]. The image shows a dissecting aortic aneurysm due to the defect. Elastin $+/-$ heterozygotes show less severe defects than the knockouts but still have altered arterial morphology and show an increased number of elastic lamellae compared to normal animals [82]. Defects in Col I lead to a variety of vascular abnormalities and can affect cardiac repair following injury [83]. (Image components from Servier Medical Art [84])

Fig. 2.

Schematic of in vitro systems for studying the effects of cECM on cell behavior. 2D systems include coating with natural ECM, tissue-derived ECM, isolated ECM functional domains, and synthetic ECM. 3D systems have incorporated natural and tissue-derived ECM as well as functional domains and synthetic ECMs. Each of these systems has provided distinct insights into the functions of the cECM. (Image components from Servier Medical Art [84])

Fig. 3.

Schematic of cECM used for cardiac therapy. Injected ECM has shown improvements in cardiac function but lacks organization and recovery of cardiac muscle mass [52–55, 85]. cECM-based cardiac patches incorporating cells have been shown to create synchronously beating structures [56]. However, modifications to natural ECM are typically required in order to match native tissue mechanical properties [57], and these structures still lack the organization of native tissue. Organized ECM structures have been created through 3D printing modalities and have led to improved cardiac differentiation [60] and mechanical properties [61]. 3D-printed tissues have also been created with micron-scale resolution and tissue organization [62]. Thickness of these tissues, as well as of more complex heart structures is limited by vascularization. However, a whole 3D printed embryonic chick heart has been printed with a soft biomaterial but is not yet able to support cells [69]. (Image components from Servier Medical Art [84])