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JACC FOCUS SEMINAR: EXTRACELLULAR MATRIX IN CARDIOVASCULAR HEALTH AND DISEASE

JACC FOCUS SEMINAR

Basic Biology of Extracellular Matrix in the Cardiovascular System, Part 1/4

JACC Focus Seminar

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ABSTRACT

The extracellular matrix (ECM) is the noncellular component of tissues in the cardiovascular system and other organs throughout the body. It is formed of filamentous proteins, proteoglycans, and glycosaminoglycans, which extensively interact and whose structure and dynamics are modified by cross-linking, bridging proteins, and cleavage by matrix degrading enzymes. The ECM serves important structural and regulatory roles in establishing tissue architecture and cellular function. The ECM of the developing heart has unique properties created by its emerging contractile nature; similarly, ECM lining blood vessels is highly elastic in order to sustain the basal and pulsatile forces imposed on their walls throughout life. In this part 1 of a 4-part *JACC* Focus Seminar, we focus on the role, function, and basic biology of the ECM in both heart development and in the adult.
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As recently as the 1980s, the extracellular matrix (ECM) was considered an inert and static scaffold. Motivating this *JACC* Focus Seminar series, ECM is now understood to be an active and dynamic tissue component regulating multiple processes including cell migration, progenitor cell self-renewal and differentiation, tissue growth and morphogenesis, fibrosis, and other processes (**Central Illustration**) (1–4). Moreover, ECM is a key element of the pathobiology of numerous cardiovascular disease processes.

In this 4-part *JACC* Focus Seminar titled "Extracellular Matrix in Cardiovascular Health and Disease," we review the full breadth of ECM in the cardiovascular system (**Table 1**). This series should serve as a benchmark and resource for basic scientists and clinicians alike. Here, in part 1 we cover basic ECM biology in development and the adult, including its

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ABBREVIATIONS AND ACRONYMS

ADAMTS = a disintegrin and metalloprotease with thrombospondin motifs

BM = basement membrane

ECM = extracellular matrix

EndMT = endothelial to mesenchymal transition

ErbB = erythroblastic oncogene B

GAG = glycosaminoglycan

HA = hyaluronan

LOF = loss-of-function

MMP = matrix metalloprotease

P = postnatal day

scRNAseq = single-cell ribonucleic acid sequencing

SLRP = small leucine rich proteoglycan

SMC = smooth muscle cell

VEGF = vascular endothelial cell growth factor

function, classification, normal biology, homeostatic turnover, and role in aging.

ECM IN THE CARDIOVASCULAR SYSTEM: A DEVELOPMENTAL PERSPECTIVE

ECM composition is tissue-specific and varies within a single tissue across development, homeostasis, and disease. Passive and active biomechanical forces inevitably involve and are distributed through the ECM to directly influence cell signaling and function, referred to as mechanotransduction (5). The cardiovascular ECM is formed from filamentous and sheet-forming protein polymers such as collagens, elastins, fibulins, and laminins. Glycosaminoglycan (GAG) polymers include hyaluronan (HA), chondroitin sulfate, heparan sulfate, and keratan sulfate, with the sulfated forms becoming linked to proteoglycan core proteins before their secretion. Chondroitin sulfate proteoglycans, including versican and aggrecan, associate with HA and cartilage link protein 1, forming aggregates that have roles in ECM hydration (6). Heparan sulfate proteoglycans, including perlecan, bind to a range of growth factors and cytokines, modifying their functions (7,8). Bridging proteins such as fibronectin, proteoglycan link proteins, periostin, and fibulins facilitate and stabilize interactions between ECM molecules and ECM cellular receptors including integrins (5). A variety of collagen cross-linking proteins modify filamentous ECM, and members of the matrix metalloprotease (MMP) and a disintegrin and metalloprotease with thrombospondin motifs (ADAMTS) families are responsible for ECM cleavage, facilitating ECM dynamics and turnover (9).

Using model organisms, many mutations in cardiovascular ECM genes have been characterized and shown to have no phenotype during development, highlighting a high degree of functional redundancy and ECM network compensation (9). However, a subset of ECM genes is essential for cardiovascular morphogenesis and integrity; next we illustrate several specific examples.

ECM IN VASCULAR DEVELOPMENT. The developing vascular system forms by vasculogenesis, the de novo generation of cord-like vascular structures from endothelial progenitors (angioblasts). This primitive vessel network expands by angiogenesis, whereby new vessels form by sprouting of pre-existing vascular beds. Within established vessels, quiescent endothelial cells are associated with a basement membrane (BM) formed predominantly from laminins, collagens IV and XVIII, perlecan, and other ECM components (**Figure 1D**). During angiogenic remodeling, endothelial cells break the BM and migrate into surrounding tissues interacting with interstitial ECM components including fibronectin, vitronectin, thrombospondins, tenascins, and different collagens (10). Depending on the vessel state, a spectrum of different ECM receptors (mainly integrins) are expressed on endothelial cell membranes.

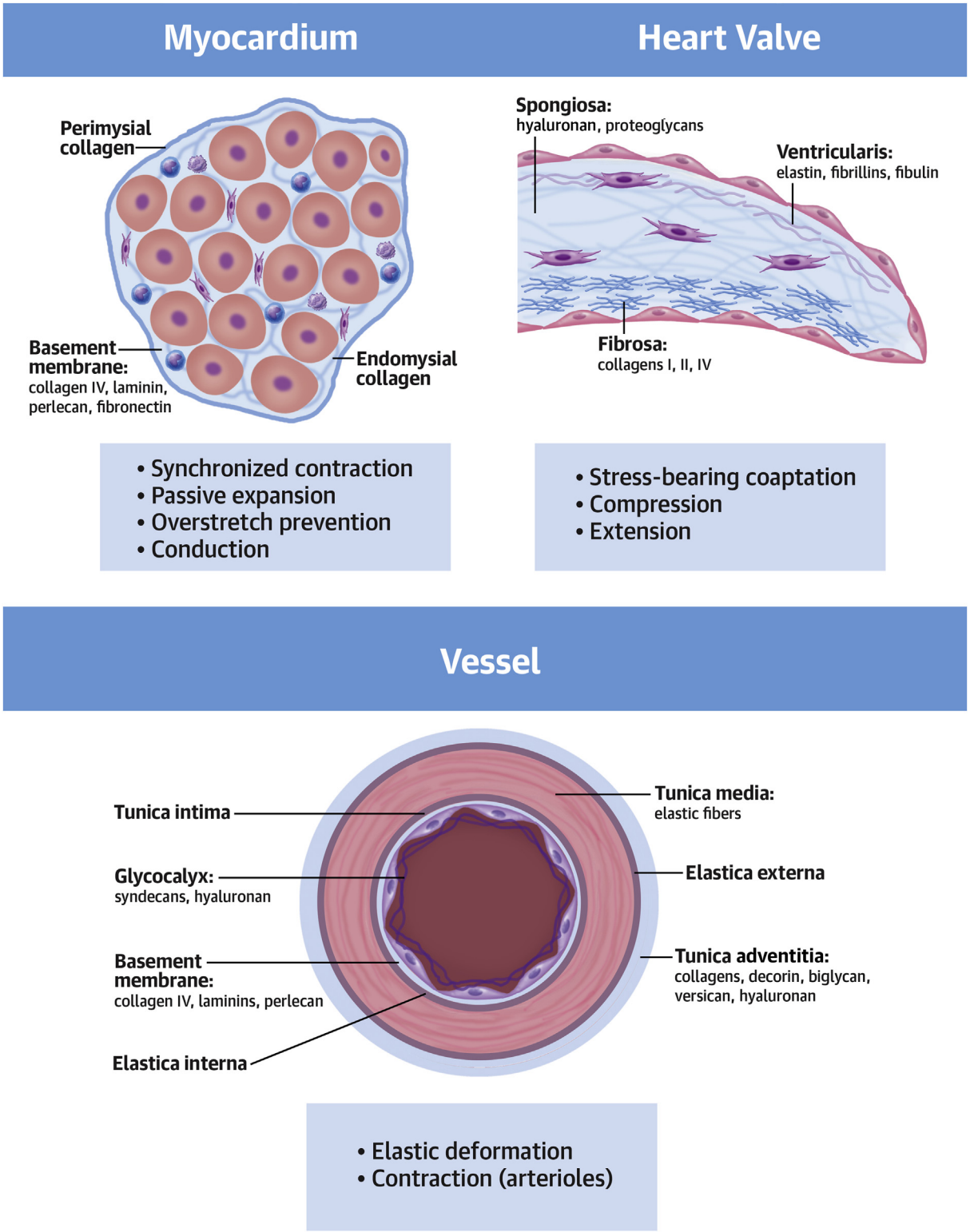
Most mutant models for ECM components and receptors do not show developmental angiogenic defects (11,12). However, germline deletion of the fibronectin gene, and deletion of alternatively spliced extra type III molecule A and B exons show defects and hemorrhage in multiple vascular beds (13). Mutation of α4/α5 and β1 integrin chains, constituting fibronectin receptors, also show early embryonic lethality associated with vascular and heart defects (14,15). Laminin α4 mutants show disrupted capillary BMs and hemorrhage at fetal stages (16), whereas fibulin 4 mutants show aortic narrowing and tortuosity due to the presence of abnormal rod-like elastin filaments (17).

As vessels remodel and enter new territories, the stiffness of the ECM around vascular projections changes, and the associated changes to

HIGHLIGHTS

- The ECM is the noncellular component of tissues throughout the body and is formed of filamentous proteins, proteoglycans, and glycosaminoglycans, which extensively interact and whose structure and dynamics are modified by cross-linking.
- Whereas historically ECM was thought to be static, thereby providing the "mortar" for cells, on the contrary, it is now recognized that the ECM is highly dynamic during development and in response to physiologic and pathophysiologic stimuli, conferring very specific signaling and mechanical functions.
- By leveraging novel tools such as scRNAseq and high-throughput proteomics, it is anticipated that a great deal more remains to be discovered, which may lead to future novel therapeutic opportunities.

CENTRAL ILLUSTRATION **Organization of Cardiac and Vascular Extracellular Matrix**



del Monte-Nieto, G. et al. J Am Coll Cardiol. 2020;75(17):2169–88.

Cardiovascular tissues face different hemodynamic and mechanical requirements that have resulted in a unique building plan and highly specialized extracellular matrix (ECM) and cellular compositions. Prominent substructures and ECM components are indicated.

TABLE 1 Overview of 4-Part *JACC* Focus Seminar: Extracellular Matrix in Cardiovascular Health and Disease

Part 1	Basic Biology of Extracellular Matrix in the Cardiovascular System • Developmental perspective • Normal biology, cellular sources, and homeostatic functioning of ECM in the adult
Part 2	Extracellular Matrix in Vascular Disease • The composition of the vascular ECM • ECM remodeling, arterial stiffness, and atherosclerosis • ECM and aortic aneurysm • Proteomics of the vascular ECM
Part 3	Myocardial Interstitial Fibrosis In Nonischemic Heart Disease • Histological basis of myocardial interstitial fibrosis • Cellular and molecular mechanisms of myocardial interstitial fibrosis • Clinical consequences of myocardial interstitial fibrosis • Diagnosis of myocardial interstitial fibrosis • Treatment of myocardial interstitial fibrosis
Part 4	Extracellular Matrix in Ischemic Heart Disease • ECM in the infarcted heart • ECM in chronic ischemic cardiomyopathy • Therapeutic opportunities: targeting the ECM in ischemic heart disease

ECM = extracellular matrix.

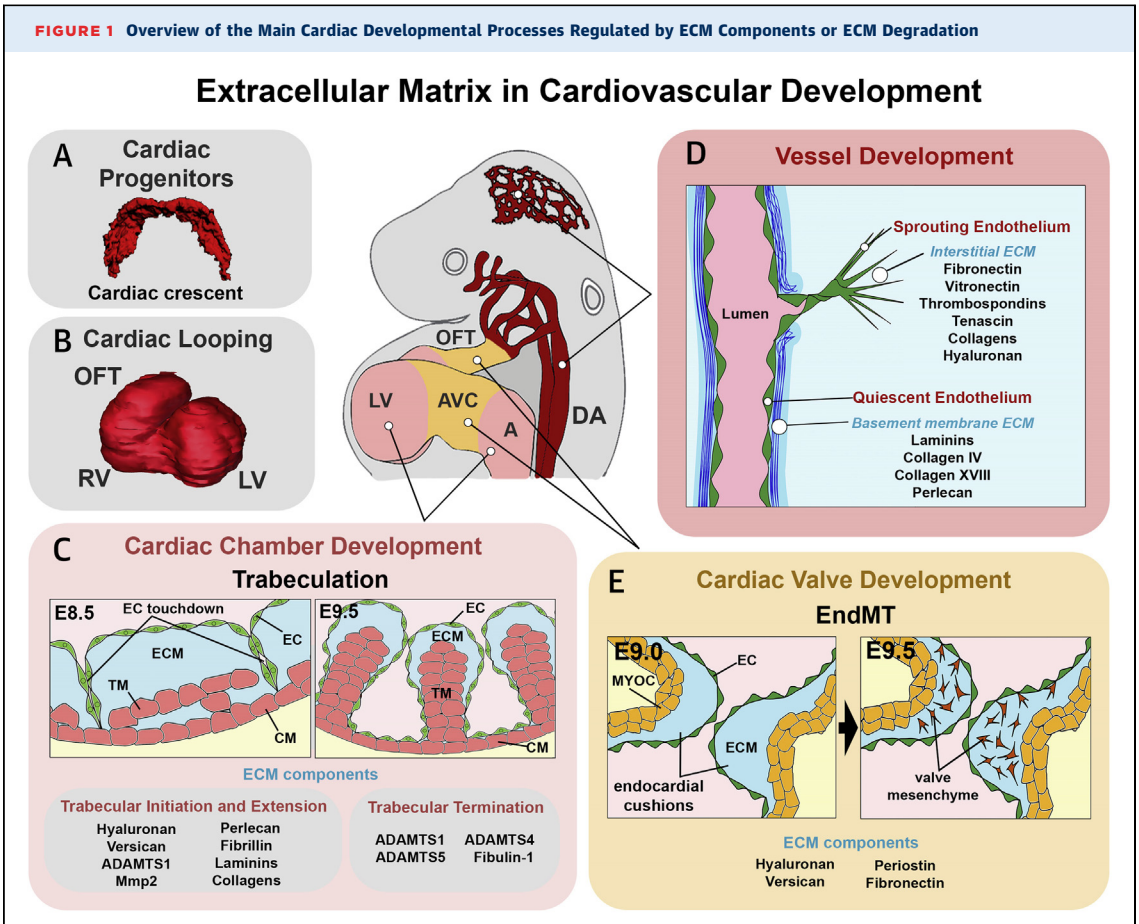
mechanotransduction can alter the activity of transcription factors such as GATA2 that regulate vessel integrity, growth, metabolism, and morphogenesis (18). ECM-integrin interactions and heparan sulfate have been linked to activation of angiokines such as vascular endothelial cell growth factor (VEGF), as well as the localization of vascular adhesion receptors such as VE-cadherin (19,20). In zebrafish, mutation of the transmembrane protein Tmem2, which degrades HA, led to excessive accumulation of HA in the heart and around vessels, blocking VEGF signaling and angiogenic development (21). This phenotype was rescued by enforced degradation of HA, or delivery of oligomeric HA or VEGF-C to embryos, suggesting a model in which dynamic degradation of HA augments VEGF signaling, perhaps by liberating VEGF molecules from the ECM or facilitating ligand-receptor interactions.

CARDIAC PROGENITOR CELL MIGRATION AND HEART TUBE DEVELOPMENT. During formation of the primitive vertebrate heart tube, myocardial and endocardial progenitors condense anteriorly. In amniotes, this region is called the cardiac crescent (**Figure 1A**). Cardiac progenitors lie adjacent to the anterior endoderm separated by a highly hydrated embryonic ECM. Bilaterally organized cardiac progenitor cells then migrate toward the embryonic midline and fuse to give rise to the primary heart tube composed of myocardial and endocardial layers separated by a thick ECM called cardiac jelly.

Studies in chick, mouse, and zebrafish embryos show that progenitor migration requires fibronectin (22–24), a major modular component of cardiac ECM that binds to multiple integrins, heparan sulfate proteoglycans, collagens, and fibrins, and is implicated in cell-substratum adhesion influencing cell shape, migration, and division. Fibronectin may bind platelet-derived growth factor, which is secreted from the endoderm and acts as a guidance molecule for the medial migration of cardiac progenitors (25). In zebrafish, fibronectin secreted from the endocardium is deposited at the midline and around cardiomyocyte progenitors as they migrate medially between the endoderm and an extra-embryonic tissue layer called the yolk syncytial layer (26). Fibronectin does not have an instructive role in migration; rather, it is necessary for the epithelialization and maturation of cardiomyocyte precursors (24).

Soon after formation of the primitive heart tube, the heart undergoes cardiac looping, which transforms the approximately straight primary heart tube into an elongated looped tube (**Figure 1B**). During cardiac looping, different regions acquire more definitive character as chamber versus nonchamber myocardium and chamber-associated versus valve-forming endocardium (27). The pathways that guide heart looping have their origins at earlier developmental stages when bilateral embryonic symmetry is broken (28). Although differential ECM distribution has no instructive role during heart looping (29), the early inhibition of MMP2 activity leads to defects in heart migration or fusion (leading to cardia bifida). However, later MMP2 inhibition blocks heart looping by interrupting regional ECM degradation necessary for breakdown of the dorsal mesocardium, a transient myocardial ligament that tethers the early heart tube to more dorsal embryonic tissues (30) and that is critical for development of torsional strain during cardiac looping (31).

CARDIAC VALVE DEVELOPMENT. In newly formed hearts, the thickness of the embryonic cardiac jelly ECM is homogeneous, but regional differences subsequently arise. In valvulogenic regions (**Figure 1E**), active ECM synthesis by myocardium and endocardium and lack of ECM degradation lead to formation of prominent acellular ECM swellings between the endocardial and myocardial layers called endocardial cushions (32). Endocardial cushions act initially as primitive valves and are the primordia of the definitive heart valve leaflets as they become cellularized by endothelial to mesenchymal transition (EndMT), producing a profibrotic valve mesenchyme (32,33). Cushions also play roles in chamber septation, knitting together the different tissue elements contributing to the atrioventricular septal complex.



(A) Early cardiac progenitor formation; **(B)** cardiac looping; **(C)** cardiac chamber development and trabeculation; **(D)** vessel development; and **(E)** cardiac valve development. A = atrium; ADAMTS1 = a disintegrin and metalloprotease with thrombospondin motifs 1; AVC = atrioventricular canal; CM = compact myocardium; DA = dorsal aorta; E8.5 = embryonic day 8.5; EC = endocardium (specialized endothelial cells lining the inner aspects of the cardiac chambers); ECM = extracellular matrix; EndMT = endothelial to mesenchymal transition; LV = left ventricle; Mmp = matrix metalloprotease; MYOC = myocardium; OFT = outflow tract; RV = right ventricle; TM = trabecular myocardium.

The proper synthesis and structural organization of ECM within endocardial cushions is critical for valve development and maturation (Figure 1E). HA is the most abundant GAG in the developing heart and is involved in valvular endothelial cell proliferation and EndMT (6). HA deficiency caused by excessive degradation (34) or loss-of-function (LOF) mutation in *Has2* and *Ugdh* genes (involved in synthesis of HA and other GAGs [35,36]) lead to cushion and valve defects, whereas excess HA leads to excessive EndMT (37). HA interacts with erythroblastic oncogene B2 (ErbB2)/ErbB3, the endocardial signaling receptor for neuregulin1 involved in regulation of endocardial EndMT and cushion ECM synthesis (38), and likely regulates its function because neuregulin1 and ERBB2 mouse mutants die around embryonic day 10.5, showing severe heart defects associated with reduced cardiac cushion and ventricular chamber ECM, a phenotype similar to that of *Has2* and versican mutants (35,39–41). Versican is also involved in valve maturation (42). Interestingly, periostin, a matricellular bridging protein that promotes fibrogenesis in cardiac valves and other fibrous cardiac structures also protects the atrioventricular valve mesenchyme against inappropriate differentiation to a myocardial fate (43). Periostin also protects the outflow tract valve mesenchyme against calcification by suppressing the expression of osteogenic transcription factor Runx2 (44). In a further advance, phagocytic macrophages derived from the hemogenic endocardium were shown to be important for the remodeling of endocardial cushion ECM (45).

Valve development is a striking example of how biomechanical forces guide tissue morphogenesis. Valve endothelial cells respond to complex flow parameters by expressing the flow-dependent transcription factor Klf2, regulating downstream Notch and canonical Wnt signaling pathways in spatially distinct patterns (46,47). These govern mesenchymal cell proliferation and cushion remodeling. As the zebrafish atrioventricular valve matures, Has2 expression (governing HA synthesis) is reduced, while fibronectin expression is increased, corresponding to the transition from a hydrostatic to fibrotic environment in forming valve leaflets (48). Fibronectin deposition is thus flow-dependent and essential for valve formation.

Human pre-valvular endothelial cells were recently generated from induced pluripotent stem cells by directed differentiation (49). On stimulation with bone morphogenetic protein 2, they underwent EndMT and expressed ECM and other markers typical of the different stratification layers (fibrosa, spongiosa, ventricularis) seen during valve maturation. This system was used to study pathways contributing to mitral valve prolapse and myxomatous degeneration (49), demonstrating the promise of these cells for valve disease modeling.

CARDIAC CHAMBER FORMATION AND TRABECULATION. During heart looping, the future cardiac ventricles undergo differentiation and proliferation, promoting chamber expansion (ballooning) (**Figure 1B**) (50). Soon after, the process of ventricular trabeculation gives rise to a sponge-like specialized myocardium on the luminal side of the chamber wall that is critical for increasing pumping efficiency of the developing heart and surface area for nutrient and oxygen exchange (**Figure 1C**) (39). The trabecular network expands in size and complexity until embryonic day 14.5 in mice (39,51). Thereafter, the process of myocardial compaction is initiated, leading to simplification of the trabecular layer through its integration into the chamber wall (52).

Soon after the formation of the primitive heart tube, the regions destined to become the cardiac chambers undergo a progressive reduction of cardiac jelly (**Figure 1C**). In most reptiles, birds, and mammals, reduction of ECM in the forming atria is virtually complete; however, atrial ECM is maintained in zebrafish where it plays a role in inhibiting trabeculation (53). Historically, the main genetic studies implicating cardiac jelly in regulation of chamber development came from analysis of LOF mutants in *Has2* (35) and versican (40). Homozygotes of these mutant strains show a total reduction of cardiac jelly in forming chambers and severely defective trabeculation; however, these studies focused largely on the roles of *Has2* and versican in valve development (35,40). LOF mutation of perlecan, a heparan sulfate proteoglycan involved in formation of the cardiomyocyte BM, leads to cardiac chamber defects involving fenestration of the ventricular myocardial wall (54). Many other ECM components are expressed in the developing ventricular chambers including laminins, fibrillins, and collagens. However, no cardiac chamber phenotypes have been seen in mutant models.

Several studies have highlighted the importance of ECM degradation by metalloproteases (ADAMTS1, ADAMTS4, and ADAMTS5) (51,55) and the metalloprotease cofactor fibulin 1 (56) in the process of trabecular termination (**Figure 1C**). LOF mutants of *Adamts1* and fibulin 1 lead to a hypertrabeculation phenotype, associated with persistence of trabecular growth beyond the normal termination stage (51,56), whereas ectopic expression of Adamts1, Adamts4, or Adamts5 promotes premature termination of trabecular growth associated with total degradation of chamber ECM (51,55). These results highlight the importance of ECM degradation at the terminal stages of trabecular development and link chamber ECM to regulation of trabecular growth.

The importance of ECM dynamics during the early phases of chamber development were only recently described (39). Del Monte-Nieto et al. (39) determined that trabecular localization, architecture, and growth are regulated by a fine balance between ECM degradation and synthesis, directed from different regions of the chamber endocardium (**Figure 1C**). The restricted activity of the Notch pathway in chamber endocardium controls ECM degradation, leading to formation of numerous endocardial sprouts that tunnel through the cardiac jelly ECM to contact the myocardial layer. The distribution of endocardial sprouts creates a series of endocardial domes delimiting cardiac jelly-rich areas called ECM bubbles, within which the trabecular myocardium expands. Formation of trabeculae likely also involves the extrusion of cardiomyocytes from the outer compact layer, as seen in zebrafish (57); however, the specific architecture of the trabecular projections in mammals is determined by endocardial cell sprouting behaviors leading to formation of endocardial domes and ECM bubbles. ECM degradation regulated by the Notch pathway occurs through transcriptional control of metalloprotease genes including *Adamts1* and is counterbalanced by ECM synthesis regulated by neuregulin1, also expressed in endocardium but

acting through its Erbb2/Erbb4 receptor complex on trabecular cardiomyocytes to promote expression of Has2 and versican at the trabecular tips. This finely regulated molecular control of ECM dynamics during trabeculation is governed by the opposing roles of the Notch and neuregulin1 pathways. Disruption of ECM degradation by mutation of *Notch1* leads to a lack of endocardial sprouting, excess ECM, and disorganized trabecular myocardial growth, whereas disruption of ECM synthesis by mutation of neuregulin1 leads to reduction of ECM and arrest of trabecular growth (39).

As trabeculation proceeds and the endocardium establishes progressively closer contact with myocardium, there is ongoing ECM degradation at the base of forming trabeculae, whereas ECM synthesis is maintained at the tips of trabeculae (**Figure 1C**). ECM degradation is dominant and progressive, eventually eliminating most of the chamber ECM at the termination phase as endocardium and myocardium become closely associated across the whole trabecular network (39,51) (**Figure 1C**). Therefore, in summary, there is a critical role for cardiac ECM as an integral component of trabecular architectural patterning and growth during chamber development (39). A summary of differing ECM molecules and components expressed during cardiovascular development, and the associated defects that arise with their perturbation (i.e., LOF), is presented in Supplemental Table 1.

EXCITATION/CONTRACTION COUPLING OF CARDIOMYOCYTES. In mammalian heart development, cardiac progenitor cells within the cardiac crescent initiate asynchronous Ca^{2+} oscillations prior to their migration to the midline. Synchronized contraction wave fronts are initiated as the heart tube is formed (58). As discussed earlier, progenitor migration is accompanied by cardiomyocyte maturation involving increased cell polarization, sarcomere assembly, and cell volume, as well as maturation of the cardiac electrical system, whose activity is essential for the maturation process (58). As the embryonic heart matures, its stiffness increases 10-fold, aligned with increases in synthesis of HA, collagens and other ECM components, and mechanosensitive adhesion complexes that include integrins, talin, and vinculin (3,59,60). The stiffness of the ECM at progressive developmental stages appears to be optimized for sarcomeric gene expression and protein assembly, as well as sarcomere spacing, connectivity and contraction, and tissue conduction (3,58). Collagen synthesis and organization is likely to be further enhanced by biomechanical strain generated within the ECM as a result of contraction. Controlled softening of the ECM by collagenase treatment, or stiffening by cross-linking, suppresses cardiomyocyte beating (3). Recently, Chiou et al. (61) proposed that in early heart development, contraction wave fronts are propagated mechanically not electrically. In their model, embryonic cardiomyocytes represent mechanically excitable inclusions in the ECM, and depolarization occurs when local biomechanical strain reaches a threshold. As such, strain and contraction "diffuse" through the system to generate the contraction wave front, before maturation of cardiomyocyte electrical coupling.

CONTROL OF FETAL CARDIOMYOCYTE PROLIFERATION BY FIBROBLAST ECM. Cardiomyocyte proliferation occurs during trabeculation and later thickening of the compact layer and is controlled by mitogenic factors secreted from both epicardium and endocardium (62). As described earlier, this process requires correct ECM dynamics (39). During later fetal development, the cardiac chamber walls become infiltrated by fibroblasts, which have their origins in the epicardium (63). Through this process, cardiomyocytes become surrounded by a connective tissue that is responsible for the synthesis and modulation of the fetal and adult cardiac ECM scaffold. Cardiac fibroblasts are recognized as having many roles as sentinels, tissue architects, paracrine signaling hubs, and lineage precursors. Fetal cardiac fibroblasts stimulated proliferation of embryonic cardiomyocytes in in vitro cocultures, whereas adult fibroblasts stimulated hypertrophy (60), highlighting the importance of fibroblast-cardiomyocyte paracrine signaling throughout heart development. Genes encoding fibronectin, collagen, tenascin C, and periostin were among those more highly expressed in fetal compared with adult fibroblasts, and synthetic matrices composed of integrin ligands such as collagen and fibronectin also stimulated cardiomyocyte proliferation, with knockdown of the genes for fibronectin and collagen3a1 reducing cardiomyocyte proliferation (60). These ECM components are in fact necessary for the activity of mitogens secreted from fetal fibroblasts, including heparin-binding epidermal growth factor-like growth factor and fibroblast growth factor (60). Downstream, fibroblast ECM components act through the cardiomyocyte-specific fetal integrin β1A isoform connecting to mitogen-activated protein kinase and phosphoinositide 3 kinase pathways. Thus, in the fetal heart, integrin ligands and other ECM components interact to coordinate the activity of cardiomyocyte mitogens secreted by fibroblasts.

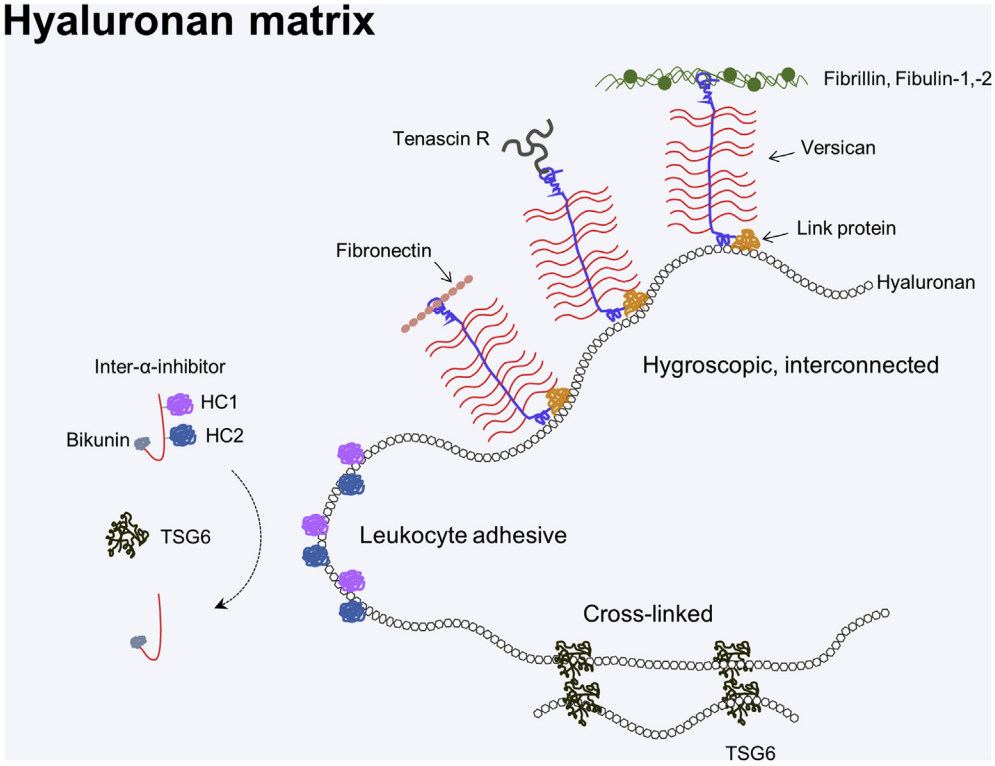
ROLE OF ECM IN FETAL DEVELOPMENT AND CONGENITAL HEART DISEASE. Human ECM gene variants have been causatively linked to congenital heart disease, generally in association with complex syndromes (9). For example, patients with mutations in the fibrillin 1 gene (*FBN1*) associated with the connective tissue disorder Marfan syndrome, show thickening of the atrioventricular valves and mitral and/or tricuspid valve prolapse, and aortic aneurysm. Patients carrying variants in the *CHST3* and *CHST14* genes (encoding enzymes involved in sulfation of GAGs) have syndromes including ventricular and atrial septal defects, respectively. Numerous mouse ECM mutants and mutant combinations also develop congenital heart disease-like phenotypes during fetal stages, highlighting the important but sometimes subtle roles played by ECM components during development (Supplemental Table 1).

ECM, POSTNATAL HEART GROWTH, AND REGENERATION. Immediately after birth, mouse cardiomyocytes retain some proliferative ability associated with a robust regenerative response to apical surgical resection or induced myocardial infarction (64). The main mechanism of cardiomyocyte renewal involves dedifferentiation and proliferation of surviving cardiomyocytes, as also seen in zebrafish hearts, which retain regenerative ability into adulthood (65). However, regenerative ability in mice wanes over the first postnatal week (64) and this is believed to be due to a decline in cardiomyocyte proliferation in favor of binucleation, reflecting the transition to hypertrophic growth (66,67). However, loss of regenerative ability occurs as early as postnatal day 2 (P2) in mice or P3 in pigs, in advance of changes in cardiomyocyte cell cycle activity (66,68). ECM is known to undergo major changes during this period and is being increasingly investigated for its roles in regeneration. There is a rapid increase in the elastic modulus of the mouse ventricle between P1 and P2 from 12 kPa to 39 kPa (66,69), which coincides with an increase in expression of ECM and cell-ECM interaction genes and proteins (66). Reduction of ECM stiffness led to a prolongation of the neonatal regenerative window until at least P3 (66), suggesting that the sharp increase in cardiac ECM stiffness postnatally is inhibitory for heart regeneration. However, periostin, a matricellular protein that contributes to the viscoelastic properties of tissues through its role in promoting ECM synthesis and fibrillogenesis (43,70), is also essential for neonatal cardiac regeneration by constraining glycogen synthase kinase 3β signaling, impacting cardiomyocyte proliferation, angiogenesis, and monocyte recruitment (71).

Agrin is a large ECM heparan sulfate proteoglycan that is implicated in self-renewal, proliferation, and differentiation of a variety of cell types (72). Agrin is expressed in mammalian fetal hearts but is downregulated during the first postnatal week (64), and fetal hearts lacking agrin show higher cardiomyocyte contraction frequencies, a phenotype that is reversed by recombinant agrin (73). In this setting, agrin binds to the α3 subunit of the membrane Na, K-adenosine triphosphatase, inhibiting its function (73). In a screen for proregenerative ECM components, agrin peptides were found to be enriched in cell-free ECM preparations of P1 hearts that were capable of stimulating proliferation of normally quiescent P7 cardiomyocytes (72). Genetic studies showed the importance of agrin for cardiomyocyte cell cycle activity and heart regeneration in the immediate neonatal period (72,73). Furthermore, direct injection of a recombinant agrin peptide into the neonatal heart extended the window of postnatal regeneration until at least P7 (72). The receptor for agrin in this context is dystroglycan 1, a component of the membrane-localized dystroglycan complex, which links ECM to the actin cytoskeleton. Binding of agrin to dystroglycan 1 stimulates extracellular signal-regulated kinase signaling and partial disassembly of the dystroglycan complex, leading to release from the membrane of the transcription factor Yap, a central transcriptional mediator of cell growth and organ size (72). In nondividing cardiomyocytes, Yap is normally tethered to the membrane dystroglycan complex via dystroglycan 1, sequestering it away from the nucleus (74). Recombinant agrin can also promote regeneration of adult murine hearts through stimulating cardiomyocyte proliferation (72), which may have significance for cardiac regenerative medicine.

SUMMARY OF THE ROLE OF ECM IN CARDIOVASCULAR DEVELOPMENT. These vignettes are by no means comprehensive, however, they serve to highlight the indispensable role of ECM during heart and vessel development. Indeed, as already discussed, the importance of ECM in early cardiovascular development has only been fully appreciated in the last 2 decades, when we have moved from the perspective of a static role as an inert scaffold that maintains tissue turgor, to a dynamic role that includes regulating critical cell and tissue functions. This is a theme that will be revisited throughout this review series.

FIGURE 2 **HA and HA-Binding Proteins**



Hyaluronan (HA) matrix diversification via HA-binding proteins highlights its multifunctionality. HA's function is diversified by HA-binding proteins that specifically bind via a link module domain. For example, interaction with versican, a large chondroitin sulfate proteoglycan can lead to large hydrated aggregates that allow cell migration and proliferation. Versican can also bring together other extracellular matrix (ECM) components (e.g., fibronectin, tenascin-R, and microfibrils) via its C-terminal domain (106). During inflammation, HA can be covalently modified by the addition of heavy chain 1 (HC1) or -HC2 from inter-α-inhibitor, a process mediated by tumor necrosis factor–stimulated gene 6 (TSG6), resulting in an ECM that is highly adhesive for leukocytes. HA can also be cross-linked by TSG6 resulting in a more condensed ECM, of which the full implications are unknown (76).

NORMAL BIOLOGY, CELLULAR SOURCES, AND HOMEOSTATIC FUNCTIONING OF ECM IN THE ADULT

In the adult, ECM is a complex, dynamic, and multicomponent network that is critical for the proper functioning of differing cardiovascular tissues and organs and that confers specific mechanical functions in response to different physiologic and pathophysiologic stimuli (**Central Illustration**). Mechanical stress, wall tension, shear stress, and pressure gradients in different vascular beds are important regulators of ECM composition and supramolecular structure. In addition, ECM signals to cardiovascular cells through specific receptors such as integrins or CD44 and provides binding sites for growth factors and cytokines. This signaling is thought to critically determine the function of cardiovascular cells such as fibroblasts and even cardiomyocytes. Many pathologies such as atherosclerosis, diabetes, pressure overload, and ischemic heart disease, as well as aspects of aging, lead to and are partly driven by pathophysiologic ECM remodeling.

Cardiac and vascular ECM networks in the adult contain collagens, various proteoglycans, matricellular proteins, and GAGs such as HA. For simplicity, ECM is often divided into structural ECM (e.g., collagens and elastin) and nonstructural ECM. However, different aspects of collagen fibril formation such as fibrillogenesis, alignment of collagen fibrils, lateral fusion, and collagen density are fine-tuned by nonfibrillar collagens and collagen-binding proteoglycans. Similarly, the HA matrix network depends on the integration of HA binding proteins (hyaladherins) such as versican. Therefore, many of

the so-called nonstructural ECM components critically affect ECM structure.

Another level of structural and likely functional complexity arises from glycosylation patterns of the cardiac and vascular glycoproteins and proteoglycans. The best-known ECM receptors are the integrin heterodimers that are activated on engagement of ECM ligands and that signal through focal adhesions into cells (outside-in signaling). Other ECM receptors include discoidin domain receptors that recognize collagen, CD44 that recognizes osteopontin, HA and versican as ligands, and Toll-like receptors that recognize ECM fragments after damage and ECM degradation. Furthermore, ECM molecules can be linked covalently or noncovalently. Covalent cross-linking is initiated by transglutaminases, lysyl oxidases, lysyl oxidase-like enzymes, and lysyl hydroxylases (75). HA can be cross-linked by tumor necrosis factor–stimulated gene 6 and pentraxin 3 (76) (**Figure 2**). Enhanced collagen cross-linking is associated with increased ventricular and vascular stiffness (77). These modifications change both mechanical and signaling properties.

Building on these themes, in this section of this review, important functions of adult cardiovascular ECM in physiological conditions are addressed. Furthermore, we point out new opportunities to explore ECM function using single-cell ribonucleic acid sequencing (scRNAseq) and other approaches.

NORMAL BIOLOGY AND CLASSIFICATION OF ECM IN THE CARDIOVASCULAR SYSTEM.

Heart. In the heart, cardiomyocytes are interconnected by intercalated disks to form a multicellular syncytium allowing coordinated myocardial excitation and contraction. Cardiomyocytes are enclosed by a network of BM proteins including networks of collagen IV, laminin, perlecan, and fibronectin (**Central Illustration**). Cardiomyocytes and endomysial fibroblasts sense the ECM network through integrin ECM receptors, thereby receiving signaling cues regulating differentiation, proliferation, migration, and excitation. Endomysial and perimysial collagens provide a structured microenvironment to cardiomyocytes, imparting stiffness to the left ventricular wall and supporting force transmission (78). This is also reflected by the fact that collagen is the most abundant ECM component in the heart. Changes in cardiac collagen networks have profound effects on myocardial contraction, relaxation, and diastolic stiffness (79), as well as electrical conduction (80). The collagenous ECM of the endomysium and perimysium also serves as a scaffold for noncardiomyocytes such as microvascular endothelial cells and fibroblasts. Collagen type I is the main collagen and confers tensile strength. Thin fibers of collagen type III contribute more to elasticity of the cardiac ECM (81). Collagen is secreted by fibroblasts as a soluble precursor molecule that is cleaved at both the N-terminus and C-terminus by specific proteinases as a prerequisite for fibrillogenesis. Collagen type I is a triple helical protein that self-assembles into fibrils in the presence of fibronectin, integrins, and collagen V. The fibril assembly occurs extracellularly at the plasma membrane, which enables cells (i.e., cardiac fibroblasts) to tightly control fibrillogenesis. Importantly, ~50 collagen-binding proteins are known, which are presumably used to form different fibril patterns (82).

Cardiac valves. Cardiac valve leaflets consist of 3 layers—the ventricularis, spongiosa, and fibrosa—that are surrounded by a layer of valvular endothelial cells and populated with valvular interstitial cells (**Central Illustration**). Each layer has a unique ECM composition to facilitate its specific function. The ventricularis layer, nearest the inflow, is predominantly composed of elastic fibers, consisting of an elastin core surrounded by a microfibril sheath containing fibrillins 1 and 2 and fibulins. This imparts flexibility to stretch and recoil during the cardiac cycle (83). The middle spongiosa layer is mainly composed of HA (84) and proteoglycans—versican, decorin, and biglycan (85). HA and proteoglycan matrices cushion blood pressure forces, assist in realignment of collagen and elastin fibers, and resist delamination (86). The fibrosa layer, nearest the outflow, is rich in fibrillar collagens I, III, and V, which impart stiffness and ensure leaflet integrity (83). Collagen type I is the predominant collagen and is mostly restricted to the fibrosa, whereas collagen III is more widely expressed. Additionally, a subendothelial BM layer is present, which consists of collagen types I and IV, as well as laminin. There are also minor ECM components expressed throughout the leaflet including vitronectin and fibronectin (87), as well as osteonectin and periostin (83,88). Recently, proteomic mapping of 16 different heart regions indicated that valves have the highest percentage of protein expression dedicated to ECM as compared to other heart regions (89).

Blood vessels. In blood vessels, tissue morphology and ECM composition are adapted to fulfill specific functions in different vascular beds: large elastic arteries (aorta and great vessels), muscular arteries and arterioles, capillaries, and venules and veins (90). The generic building plan of blood vessels consists of the tunica intima, tunica media, and tunica adventitia (**Central Illustration**). The layers are connected by the

membrana elastica interna and membrana elastica externa. The tunica intima represents the luminal lining of endothelial cells that are attached to the BM. The tunica media contains contractile smooth muscle cells (SMCs) and allows active vasoconstriction and relaxation, whereas the tunica adventitia attaches the vessel with the surrounding connective tissue. Whereas in contractile arteries, the tunica media is thickest, in veins, the tunica adventitia is most pronounced.

Endothelial glycocalyx extends from the endothelial surface into the lumen (**Central Illustration**). It plays a protective role by supporting endothelial barrier function, preventing platelet adhesion, and facilitating the rolling of immune cells on the endothelial surface (91). A variety of pathophysiologic stimuli cause glycocalyx shedding, which is likely among the first steps in immune cell extravasation and thrombotic complications (92). Endothelial glycocalyx is composed of heparan sulfate GAGs (syndecans) and HA (93). In addition, the polyanionic glycocalyx traps a variety of heparin-binding proteins and hyaladherins, as well as cationic proteins.

The BM of blood vessels is composed of collagens type IV and XVIII, laminins, nidogens/entactins, and perlecan. Characteristically, von Willebrand factor is present in the endothelial BM, which initiates platelet adhesions and blood coagulation via factor VIII when the endothelial layer becomes disrupted. Collagen IV plays a key role in ensuring BM stability. Laminins, in particular laminin 411, are also critical constituents of the BM in forming networks. The 2 networks (laminin and collagen IV) are connected by nidogens. Perlecan stabilizes BMs by interactions with the above-mentioned ECM constituents. In addition, perlecan interacts with heparin-binding growth factors and cytokines and protects the BM from proteolytic degradation. The ECM of BM signals through integrins to endothelial cells and regulates pivotal endothelial functions such as adhesion molecule expression, tight junction formation, endothelial metabolism, and prostacyclin synthesis. The BM is impermeable to cells, with the exception of leukocytes.

The tunica media provides elasticity to arteries, facilitating their pulsatile stretch. Therefore, the aorta and great vessels contain large amounts of elastic fibers, whereas more distal arteries have pronounced SMC layers. Elastin networks with intervening SMCs are assembled concentrically, which allows an active regulation of vascular tone and luminal diameter. In muscular arteries, elastin is not deposited as sheets but more in the form of fibers. In contrast, capillaries have no regular tunica media but instead a sheet of scattered pericytes that are encapsulated in BM.

In the tunica adventitia, a loose ECM is formed containing collagens, collagen-binding proteoglycans (e.g., biglycan and decorin), and HA. In addition, large amounts of versican, a HA-binding proteoglycan, are present. The adventitia also harbors vasa vasorum, fibroblasts, stem cells, and immune cells.

TYPICAL ECM COMPONENTS OF HEALTHY MYOCARDIUM, HEART VALVES, AND BLOOD VESSELS. Collagens. Collagens of the heart can be subdivided into fibrillar and nonfibrillar collagens. Major fibrillar forms are collagen types I, III, V, and XI (94). Collagen types I and III are the main collagens in the heart and blood vessels (81). Collagen type I forms thick rod-like fibers (50- to 150-nm diameter) conferring high tensile strength. Certain mutations of collagen type I alter diastolic compliance, such as in osteogenesis imperfecta mice. On the contrary, fine fibrils used for highly flexible reticular networks are built from collagen type III, which is typical also for skin, fetal tissue, and blood vessels. Collagen type III is mixed with type I in the heart and the type I-type III ratio influences mechanical properties. Accordingly, the proportion of type III is highest in the epimysium and decreases in the perimysium. In addition, the proportion of type I collagen increases with age, which contributes to ECM stiffening (81). Collagen type V is found in the interstitium and regulates collagen fibrillogenesis, whereas collagen type XI has the same function in heart valves. The nonfibrillar collagen type IV forms an open network with other ECM molecules of the BM and is important for BM cell adhesion and molecular transport. Collagen type VIII is expressed at low levels and might contribute to connecting the BM with elastic laminae and microfibrils and the elastic laminae with collagen fibrils. Collagen type VI is expressed in the adult heart and in the media and adventitia of blood vessels, although its functions are not well understood. Collagen type XV is indispensable for normal cardiac function as shown by knockout mice that develop vascular permeability, perturbed cardiomyocyte alignment, and abnormal interstitial ECM formation resulting in cardiomyopathy. The role of collagen type XVIII in the cardiovascular system is not well understood (81,95).

Laminins. Laminins are glycoproteins that are required for BM assembly and function both in the heart and vasculature. Laminin is assembled from various α-, β-, and γ-chains that form 15 of the 60 possible heterotrimers. The chains are covalently linked to stabilize the heterotrimer. The 3 short arms and a long coiled-coil structure formed from all 3 chains to create a cross-like molecule of 700 to

900 kDa (96). Laminin isoforms form networks and through their integrin-binding domain mediate cell adhesion (i.e., ligand for α3β1, α6β1, α6β4, α7β1 integrins) (97).

Elastin. Elastin is synthesized as tropoelastin, which is processed extracellularly to elastin. Supramolecular elastin aggregates are then cross-linked by lysyl oxidases at lysine-rich regions. Elastic fibers consist of an elastin-rich core surrounded by a sheath of microfibrils composed of several components including fibrillin 1. This elastin can be extended more than 2-fold, which is important to enable arteries to extend and retract in response to arterial pulse waves and thereby pushing blood forward in diastole. In addition, elastin and polymeric collagen inhibit SMC proliferation in the vessel wall. Interestingly, fibrillin 1 mutations cause Marfan syndrome that manifests by dilation and dissection of large arteries. Similarly, the fibulins and elastin microfibril interfacers are critical for proper formation of the elastin network in the vessel wall, for example, fibulin 5 null mice have tortuous and elongated aortas (98).

Fibronectin. Fibronectin is a glycoprotein secreted by many cell types including cardiac fibroblasts and endothelial cells, and it can act as a template for assembly of fibrillin 1 (99) and latent transforming growth factor-β-binding proteins (100). After integrin-dependent polymerization, fibronectin is required for collagen fibril assembly (82,101). Furthermore, fibronectin activates important cellular responses through integrin signaling (α5β1 and αv-class heterodimers) such as adhesion, proliferation, migration, and differentiation.

Hyaluronan. HA is linear glycosaminoglycan composed of alternating β-(1,4)-N-acetyl-D-glucosamine- and β-(1,3)-D-glucuronic acid-containing disaccharides. It is synthesized at the plasma membrane by HA synthases and is not modified by sulfation or acetylation. Therefore, HA is identical in all mammals and evolutionarily lower animals. It is an important component of the microenvironment and is of extremely high molecular mass (up to 7 MDa) and large hydrodynamic volume. HA is a main component of adventitial and valve ECM. HA binds to several receptors at the surface of mesenchymal and immune cells. The best-known HA receptors are CD44, receptor of HA-mediated motility, and lymphatic vessel endothelial HA receptor 1. These receptors regulate the phenotypes of fibroblasts, immune cells, endothelial cells, and SMCs (102–104). Furthermore, the binding of different proteins and ECM constituents to HA, such as HA-binding proteoglycans versican or tumor necrosis factor-stimulated gene 6, modify the HA matrix and skew it into different functions (Figure 2).

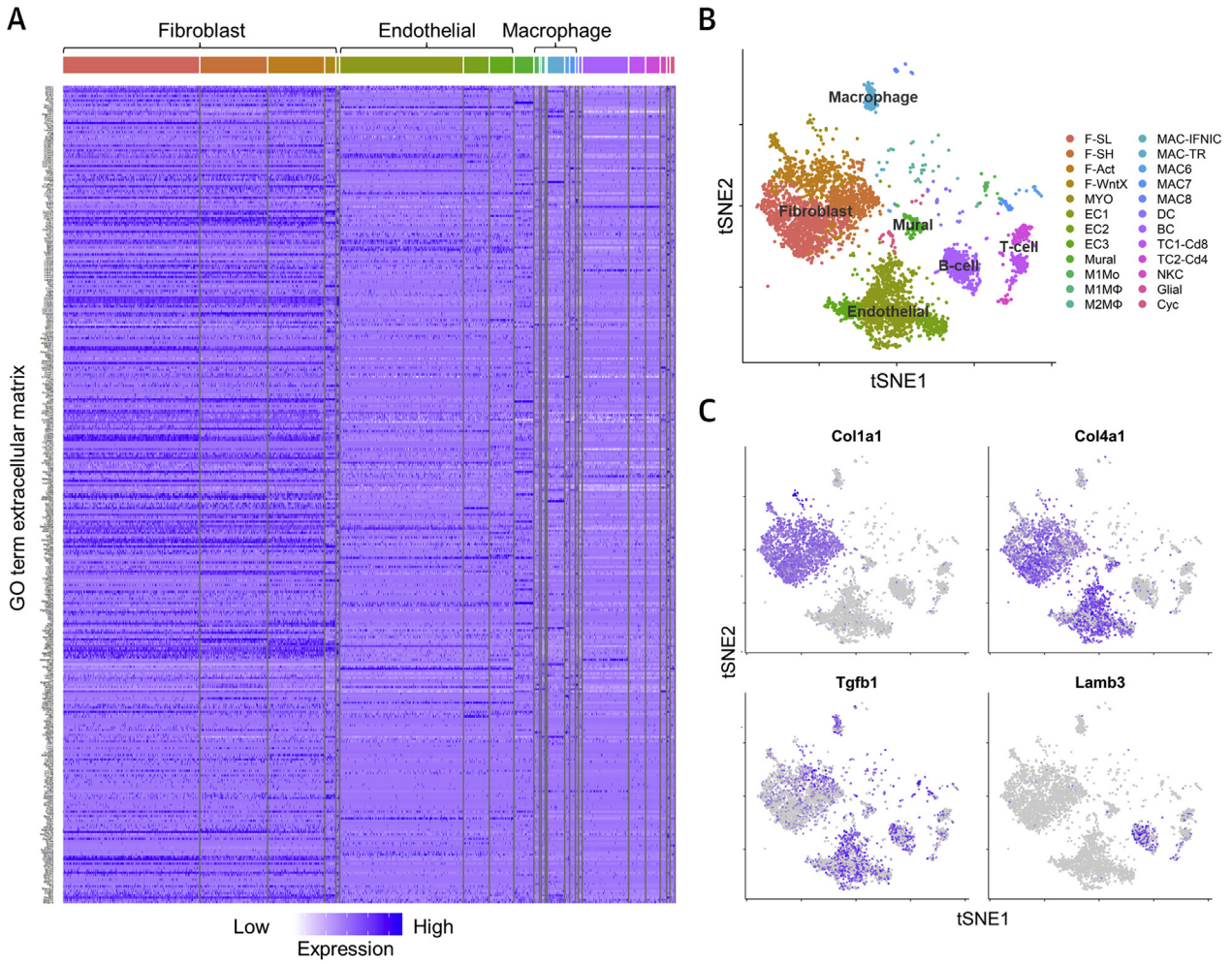
Proteoglycans. ***Cell surface proteoglycans.*** Syndecans 1 to 4 are transmembrane heparan-/chondroitin sulfate proteoglycans with unique extracellular domains that signal through a short intracellular component in concert with growth factors and other ECM molecules (105). Functionally, syndecan is thought to modulate heparin-binding growth factor activity and has an anti-inflammatory function. Additionally, glypicans 1 to 6 are glycosyl-phosphatidylinositol-anchored heparan sulfate proteoglycans that are involved in Wnt, hedgehog, fibroblast growth factor, and bone morphogenetic protein signaling.

HA-binding proteoglycans. Versican binds through its G1 domain specifically to HA and via its G3 domain to other ECM molecules. Thereby, versican/HA complexes can form large multimolecular aggregates, which are detected mainly in the pericardium and also the adventitia of blood vessels including the coronary arteries. Versican is present as various splice variants (V0, V1, V2, V3) that vary in the number of chondroitin sulfate side chains attached to the core protein. The physiological role in the healthy adult heart and vasculature has not been defined; however, it is critical for embryonic development and response to injury in the cardiovascular system (106).

BM proteoglycans. Perlecan is the largest heparan sulfate proteoglycan and has important functions in healthy BM, being critical for BM assembly and during cardiac development. Furthermore, the heparan sulfate side chains enable multiple interactions with heparin-binding proteins such as growth factors and chemokines (107).

Small leucine rich proteoglycans. Small leucine rich proteoglycans (SLRPs) are divided into 5 different classes based on their structure. SLRPs are involved both in cardiac and vascular homeostasis and remodeling. Knowledge about their role in physiological conditions is limited and best understood for Class I and II SLRPs. The Class I SLRPs decorin and biglycan carry 1 or 2 chondroitin sulfate and dermatan sulfate side chains, respectively. Important functions include regulation of growth factor availability and activity (i.e., transforming growth factor-β1), as well as modifying the assembly of collagen fibrils and fibronectin. The loss of decorin and biglycan disturbs collagen fibrillogenesis, leading to skin fragility and bone ECM disturbance. Furthermore, the dermatan sulfate side chains of biglycan balance

FIGURE 3 **Cellular Sources of ECM Production in Cardiac Tissue Revealed by scRNAseq**



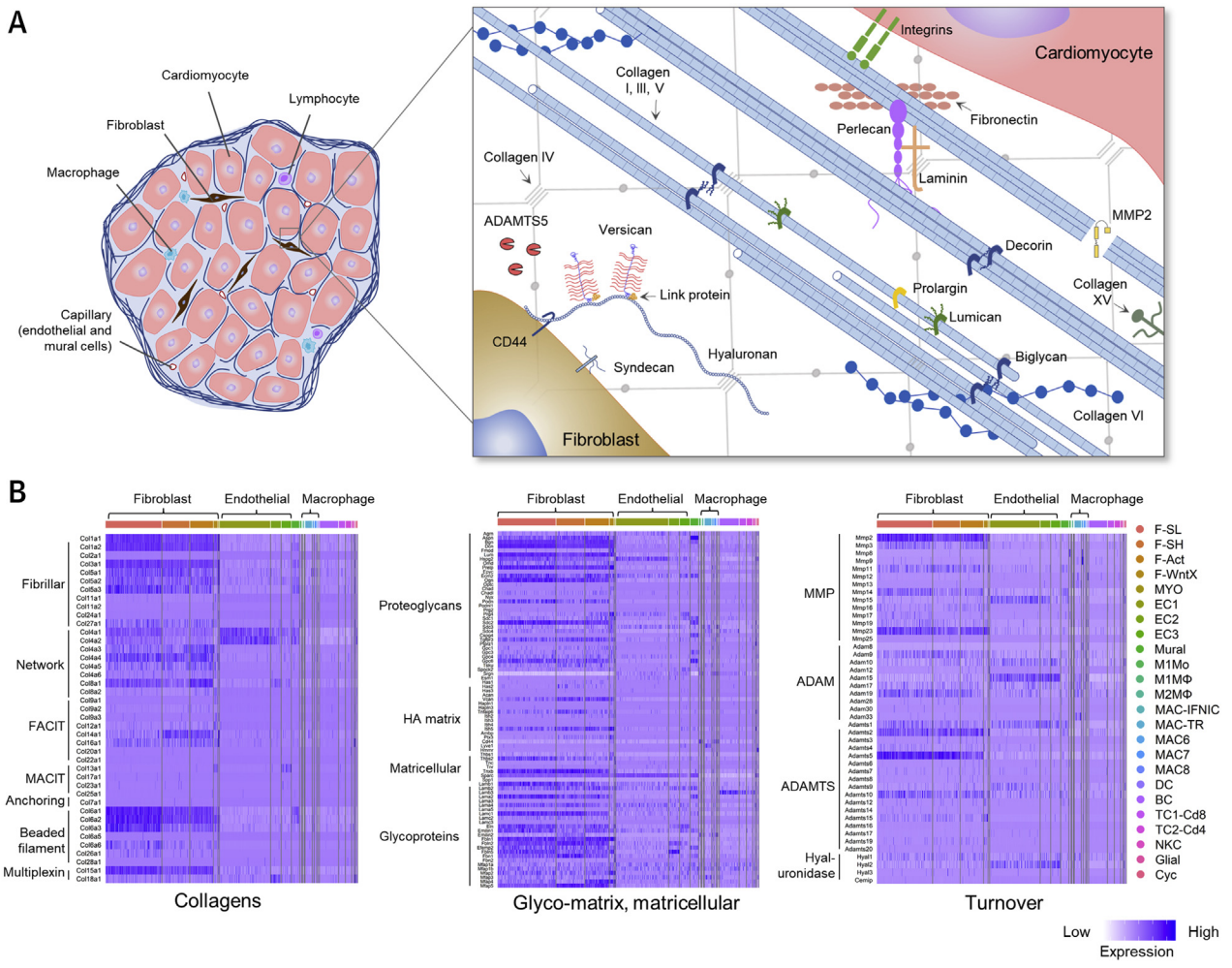
Single-cell ribonucleic acid sequencing (scRNAseq) of the total interstitial, noncardiomyocyte, cell population of control hearts. **(A)** Heat map of ECM gene ontology (GO) term expression across all populations, confirming the highest expression of ECM is generated by fibroblasts. **(B)** *t*-distributed stochastic neighbor embedding (tSNE) plot of cell population clusters showing that the major cell types, (i.e., fibroblasts and endothelial cells), can be subdivided into 5 distinct fibroblast subpopulations (fibroblast-Sca1-low [F-SL], fibroblast-Sca1-high [F-SH], fibroblast-activated [F-Act], fibroblast-Wnt expressing [F-WntX], and myofibroblast [MYO]) and 3 distinct endothelial cell (EC) populations (EC1 to EC3). **(C)** tSNE plots showing the expression patterns of collagen type I, collagen type IV, transforming growth factor-β1 (Tgfb1), and laminin subunit β3 (Lamb3). Figure generated using data made available by Farbehi et al. (119). BC = B cell; Col1a1 = α1, type I collagen; Cyc = cycling cell; DC = dendritic cell; M1Mo = M1 monocyte; M1MΦ = M1 macrophage; MAC-IFNIC = macrophage-interferon inducible cell; MAC6 = macrophage 6; MAC-TR = macrophage-tissue resident; NKC = natural killer cell; TC1-Cd8 = T-cell 1-Cd8+; TC2-Cd4 = T-cell 2-Cd4+.

thrombin activity by activation of heparin cofactor 2 (108).

Matricellular proteins. Thrombospondins 1 to 5, secreted protein acidic and rich in cysteine, tenascins, osteopontin, periostin, and the cysteine-rich angiogenic inducer 61/connective tissue growth factor/nephroblastoma-overexpressed (CCN) protein family are important positive and negative regulators in vascular and cardiac physiology. For example, thrombospondins 1, 2, and 5 are known to regulate collagen fibril assembly, whereas thrombospondins 1 and 2 are negative regulators of physiological angiogenesis and cardiac remodeling (reviewed in [109]).

CELLULAR SOURCES OF ECM IN THE ADULT CARDIOVASCULAR SYSTEM. Cells within the heart. Most of the cardiac mass is attributable to cardiomyocytes. However nonmyocyte cells outnumber cardiomyocytes, and recent data indicate

FIGURE 4 Gene Expression Profile of Major ECM and ECM-Associated Components in the Nonmyocytic Cell Populations of Healthy Adult Myocardium



(A) Graphical representation of the cellular composition and ECM organization in the myocardium. **(B)** Heat maps of ECM gene expression from scRNAseq analysis of control hearts. Figure generated using data made available by Farbehi et al. (119). Acan = aggrecan; ADAM = a disintegrin and metalloproteinase; Adam8 = a disintegrin and metalloproteinase domain-containing protein 8; Agrn = agrin; Ambp = α1-microglobulin/bikunin precursor; Aspn = asporin; Bgn = biglycan; Cemip = cell migration-inducing and hyaluronan-binding protein; Chad = chondroadherin; Chadl = chondroadherin-like; Cspg4 = chondroitin sulfate proteoglycan 4; Dcn = decorin; Ecm2 = extracellular matrix protein 2; Efemp2 = epidermal growth factor-containing fibulin-like extracellular matrix protein 2; Eln = elastin; Emilin1 = elastin microfibril interfacer 1; Epyc = epiphycan; Esm1 = endothelial cell-specific molecule 1; FACIT = fibril-associated collagens with interrupted triple helices; Fbln1 = fibulin 1; Fbn1 = fibrillin 1; Fmod = fibromodulin; Gpc1 = glypican 1; Hapln1 = hyaluronan and proteoglycan link protein 1; Has1 = hyaluronan synthase 1; Hmmr = hyaluronan-mediated motility receptor; Hspg2 = heparan sulfate proteoglycan core protein 2; Hyal1 = hyaluronidase 1; Itih2 = inter-α-trypsin inhibitor heavy chain H2; Lama2 = laminin α2; Lamb1 = laminin β1; Lamc1 = laminin γ1; Lum = lumican; Lyve1 = lymphatic vessel endothelial hyaluronan receptor 1; MACIT = membrane associated collagens with interrupted triple helices; Mfap1a = microfibrillar-associated protein 1a; Nyx = nyctalopin; Ogn = osteoglycin; Omd = osteomodulin; Optc = opticin; Podn = podocan; Podnl1 = podocan-like 1; Prelp = proline and arginine-rich end leucine-rich repeat protein; Prg2 = proteoglycan 2; Ptprz1 = receptor-type tyrosine-protein phosphatase ζ1; Ptx3 = pentraxin-related protein 3; Sdc1 = syndecan 1; Sparc = secreted protein acidic and rich in cysteine; Spock2 = SPARC (osteonectin), Cwcv and Kazal-like domains proteoglycan 2; Spp1 = secreted phosphoprotein 1; Srgn = serglycin; Tgfbr3 = transforming growth factor β receptor III; Thbs1 = thrombospondin 1; Tnc = tenascin C; Tnfalp6 = tumor necrosis factor α-induced protein 6; Tnr = tenascin R; Tnxb = tenascin XB; Tsku = Tsukushi, small leucine-rich proteoglycan; Vcan = versican; other abbreviations as in Figures 1 to 3.

that both fibroblasts and endothelial cells together are the predominant cells in the heart (110). Resident cardiac fibroblasts derive during development from the epicardium and endocardium, with a minor proportion possibly from neural crest cells. Cardiac fibroblasts reside in the endomysium and fulfill a key role in maintaining the physiologic ECM environment around cardiomyocytes (111). Elaboration of ECM by cardiac fibroblasts is regulated by mechanical, electrical, and neurohormonal stimulation (112,113). Other

TABLE 2 Overview of Enzymes and Substrates Involved in Cardiovascular ECM Turnover

Class	Type	Name	ECM Substrates
MMP	Collagenases	MMP1	Collagens (I, II, III, VII, X), gelatin, tenascin, perlecan, entactin, aggrecan, link protein
		MMP8	Collagens (I, II, III), gelatin, aggrecan, link protein
		MMP13	Collagens (I, II, III, IV, IX, X, XIV), aggrecan, perlecan, tenascin, fibronectin, osteonectin, laminin
		MMP18	Collagen I, gelatin
	Gelatinases	MMP2	Gelatin, collagens (I, II, III, IV, V, XI), vitronectin, fibronectin, laminin
		MMP9	Gelatin (III, IV, V), entactin, aggrecan, elastin, link protein, vitronectin
	Stromelysins	MMP3	Collagens (III, IV, X), aggrecan, decorin, gelatins, tenascin, link protein, perlecan, fibronectin, laminin
		MMP10	Collagens (III, IV, V), aggrecan, fibronectin, laminin, link protein
		MMP11	Collagen IV, gelatin, fibronectin, laminin
	Matrilysins	MMP7	Collagens (I, IV), gelatin, decorin, elastin, fibronectin, vitronectin, laminin, tenascin
		MMP26	Collagen IV, fibronectin, gelatin
	Membrane type	MMP14 (MT1-MMP)	Collagens (I, II, III), gelatin, aggrecan, fibronectin, fibrin, laminin
		MMP15 (MT2-MMP)	Fibrin, fibronectin, tenascin, nidogen, aggrecan, perlecan, laminin
		MMP16 (MT3-MMP)	Collagen III, gelatin, fibronectin, fibrin
		MMP24 (MT5-MMP)	Gelatin, fibronectin, proteoglycans
	GPI anchored	MMP17 (MT4-MMP)	Gelatin, fibrinogen
		MMP25 (MT6-MMP)	Collagen IV, gelatin, fibrin, SPARC
	Metalloelastase	MMP12	Collagen IV, fibronectin, laminins, vitronectin, proteoglycans, chondroitin sulfate, elastin
	Enamelysin	MMP20	Collagen V
	Other	MMP19	Collagen IV, nidogen, laminins, fibronectin
		MMP23 (CA-MMP)	Auto-proteolysis, unknown
		MMP27	Unknown
		MMP28	Unknown
ADAM		ADAM8	Fibronectin (133)
		ADAM9	Laminin
		ADAM12	Gelatin (134)
ADAMTS	Aggrecanase	ADAMTS1	Aggrecan, versican, syndecan 4, nidogens 1 and 2, gelatin
		ADAMTS4	Aggrecan, versican, biglycan, reelin, brevican, matrilin 3, oligomeric matrix protein
		ADAMTS5	Aggrecan, versican, reelin, biglycan, matrilin 4, brevican
		ADAMTS8	Aggrecan
		ADAMTS9	Aggrecan, versican, fibronectin
		ADAMTS15	Aggrecan, versican
		ADAMTS20	Versican
	Procollagen N-propeptidases	ADAMTS2	Procollagens (I, II, III, V), fibronectin
		ADAMTS3	Procollagen II, biglycan, fibronectin, LTBP1
		ADAMTS14	Procollagens I and III, fibronectin, LTBP1
	COMP-cleaving	ADAMTS7	COMP
		ADAMTS12	COMP
	vWF proteinase	ADAMTS13	vWF
	Other	ADAMTS6	Unknown
		ADAMTS10	Fibrillins 1 and 2
		ADAMTS16	Aggrecan, fibronectin
		ADAMTS17	Unknown
		ADAMTS18	Unknown
		ADAMTS19	Unknown
Hyaluronidases		Hyal1	Hyaluronan, chondroitin, chondroitin sulfate
		Hyal2	Hyaluronan, chondroitin, chondroitin sulfate
		Hyal3	Hyaluronan, chondroitin, chondroitin sulfate
		Hyal4	Chondroitin, chondroitin sulfate, hyaluronan
		Hyal5	Hyaluronan, chondroitin, chondroitin sulfate
		Hyal6	Hyaluronan, chondroitin, chondroitin sulfate
		Cemip	Hyaluronan, chondroitin, chondroitin sulfate
		Cemip2	Hyaluronan, chondroitin, chondroitin sulfate
		Spam1	Hyaluronan, chondroitin, chondroitin sulfate

References for ECM substrates can be found in the following reviews (122,128–132) unless noted in the table.

ADAM = a disintegrin and metalloproteinase; ADAMTS = a disintegrin and metalloproteinase with thrombospondin motifs; CA = cysteine array; Cemip = cell migration-inducing and hyaluronan-binding protein; COMP = cartilage oligomeric matrix protein; ECM = extracellular matrix; GPI = glycosyl-phosphatidylinositol; Hyal1 = hyaluronidase 1; LTBP1 = latent transforming growth factor-β-binding protein; MMP = matrix metalloprotease; MT1 = metallothionein 1; Spam1 = sperm adhesion molecule 1; vWF = von-Willebrand factor.

cell types within the heart include mast cells and resident immune cells such as tissue resident macrophages (114) that contribute to tissue and likely also ECM homeostasis by interactions with fibroblasts and ECM degradation (115). This homeostatic function of resident macrophages is in contrast to that of monocyte-derived macrophages that are typically involved in the response to pathophysiologic stimuli. Furthermore, prototypical vascular cells as detailed herein contribute to the cardiac ECM microenvironment in health and disease.

Vascular cells. Endothelial cells form the luminal lining in blood vessels and maintain a glycocalyx extending into the lumen and a BM at the basolateral side. In the tunica media of blood vessels, SMCs are primarily responsible for production of ECM. SMC of the tunica media are surrounded by a BM and have a differentiated contractile and nonproliferative phenotype. The contractile function depends on α-smooth muscle actin expression and interaction with collagen- and laminin-binding integrins. Their phenotype switches to a secretory and proliferative phenotype in response to growth factors, inflammatory mediators, and remodeling of the mature healthy ECM environment (elastin and collagen degradation) (116). Pericytes are found in the circumference of capillaries. Furthermore, immune cells enter and leave the vascular walls. In principle, many immune cells such as macrophages are able to synthesize and degrade vascular ECM, but to what extent this occurs in healthy conditions is not clear yet (104,117).

ScRNAseq of cardiac cells. The physiologic functions of many ECM molecules in healthy cardiovascular organs, particularly those that are less abundant, remain to be discovered. Furthermore, most ECM-related studies focus on phenotypes in disease models and not homeostasis. In addition, ECM proteomics has only recently become possible and has not been widely used to describe the ECM of healthy tissues (118). As a solution, scRNAseq appears poised to address this issue, and recently, 2 groups provided scRNAseq data from the cardiovascular systems of adult mice (119,120). We have used data from one of these (119) to explore the cardiac ECM and ECM-associated transcriptome. Importantly, this study revealed 5 different subpopulations of cardiac fibroblasts, including a population devoted to antagonizing WNT signaling (fibroblast-Wnt expressing). **Figure 3** gives an overview of the main cell populations, including subtypes of fibroblasts, endothelial cells, resident macrophages, T cells, B cells, and vascular cells, as well as proliferating cells in adult murine hearts under normal homeostasis. The heat map of ECM gene ontology terms not only shows that ECM genes are most strongly expressed in fibroblasts, but it also allows us to appreciate the vast number of genes involved in cardiac ECM. Furthermore, it demonstrates that, to some extent, almost all cardiac nonmyocyte populations contribute to cardiac ECM gene expression (**Figures 3A and B**). As expected, collagen type I expression is highest in cardiac fibroblast clusters. Collagen type IV as part of the BM is expressed by both endothelial cells and fibroblasts. Transforming growth factor-β1 as an example of an ECM-promoting growth factor is widely expressed by endothelial cells, B cells, and resident macrophages, highlighting intercell communication. This analysis can also be used to compare the expression of single ECM genes in different cell populations to stimulate new hypotheses about ECM regulation and function. For example, *Lamb3* (laminin subunit β3), appears to be expressed primarily by B cells (**Figure 3C**). A closer view on the expression patterns of the collagens and the glycomatrix is given in **Figure 4**. The expression pattern of collagens fits with the current paradigm that collagen types I, III, IV, V, and VI are the predominant collagens of healthy myocardium. Interestingly, fibroblast subpopulations show heterogeneous expression levels of collagens, (e.g., *Col6a1*, *Col6a2* and *Col6a3*) that might relate to their specific phenotypes (**Figure 4B**). Similarly, glycoproteins and proteoglycans are expressed mainly in fibroblasts; however, there is differential expression in other cell types. For example, the expression of *Lyve1*, an HA receptor, is strongly associated with tissue resident macrophages (**Figure 4B**).

HOMEOSTATIC TURNOVER AND ROLE IN AGING.

The turnover of collagenous and noncollagenous ECM is mediated by the endopeptidase MMPs and is balanced by tissue inhibitors of MMPs. In addition, other proteases contribute to ECM turnover such as those related to the ADAMTS family and glycolytic enzymes such as glycosaminoglycan-degrading enzymes (e.g., hyaluronidases). In the adult, MMPs are thought to be responsible for the majority of cardiovascular ECM turnover (primarily collagens), whereas the ADAMTS family plays a larger role in the embryo. MMPs are expressed by cardiomyocytes, fibroblasts, SMCs, and endothelial cells (121). MMP activation is achieved by removal of their N-terminal propeptide, a tightly regulated process typically catalyzed by autoproteolysis, serine proteases (e.g., plasmin), or by other MMPs (e.g., MMP3 activation of pro-MMP1) (122). A list of major cardiovascular ECM-degrading enzymes and their substrates can be found in **Table 2**. Looking back to the scRNAseq data, a unique expression pattern of ECM turnover genes among

different cell types can be appreciated (**Figure 4B**). For example, fibroblast populations are characterized by high expression of *Mmp2*, *Mmp23*, *Adamts2,* and *Adamts5*.

As a paradigm covered in depth in part 3 of this *JACC* Focus Seminar, the aged healthy heart shows progressive interstitial fibrosis and ECM stiffening, which is likely driven by age-dependent local changes such as myocyte loss and subsequent hypertrophy of remaining myocytes as well as increased peripheral vascular stiffness and increased afterload. As a consequence, left ventricular hypertrophy, impaired ventricular relaxation, and diastolic dysfunction develop that ultimately lead to heart failure with preserved ejection fraction commonly diagnosed in the healthy elderly patient.

ECM-IMMUNE INTERACTIONS. Evidence is growing that the ECM modulates immune reactions and that it may be an important modulator of immunological surveillance. During homeostasis, intact ECM does not activate immune cells or immune responses and thereby contributes to an anti-inflammatory environment. However, in response to pathophysiologic stimuli, ECM degradation and fragmentation lead to the release of ECM fragments that activate inflammatory responses either through Toll-like receptors or altered signaling properties. Furthermore, recruitment of immune cells is stimulated by matrix fragments (matrikines) (123).

The interactive networks of ECM molecules can also be remodeled to acquire different functions in health and disease. For example, HA-binding proteins, which affect migratory and proliferative phenotypes (e.g., of fibroblasts), can be recruited into the HA matrix or even transform the HA matrix into a proinflammatory ECM that retains monocytes and macrophages (**Figure 2**) (124). Of this family of HA-binding and -modifying proteins, many are expressed by cardiac fibroblasts as suggested by the scRNAseq data, including aggrecan (*Acan*); versican (*Vcan*); link proteins 1 and 3 (*Hapln1, -3*); tumor necrosis factor-stimulated gene 6 (*Tnfaip6*); inter-α-inhibitor heavy chains 2, 3, 4, and 5 (*Itih2, -3, -4, -5*); bikunin (*Ambp*); and pentraxin 3 (*Ptx3*) (**Figure 4B**). HA is also an important part of the immune synapse, thereby stimulating T-cell responses (125). Furthermore, GAG side chains of perlecan, syndecans, and Class I and II SLRPs (decorin, biglycan) activate anti-thrombin III and heparin cofactor 2, respectively (108,126), and inhibit thrombin activity, which plays an important proinflammatory role next to its role in hemostasis (127). Finally, through heparan sulfate and dermatan sulfate GAGs, intact ECM is able to store growth factors and cytokines that can further modulate immune-related and other processes.

CONCLUSIONS

The last 2 decades have seen enormous advances in our understanding of ECM physiology and pathobiology. Historically, ECM was thought to be static thereby providing the "mortar" for cells, but it is now recognized that the ECM is highly dynamic in response to physiologic and pathophysiologic stimuli and confers very specific signaling and mechanical functions. These important paradigm shifts have seen many new insights arise in both development and in the adult, however, by leveraging novel tools such as scRNAseq and high-throughput proteomics, it is anticipated that a great deal more remains to be discovered.

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KEY WORDS cardiovascular, collagen, development, homeostasis, matrix

APPENDIX For a supplemental table and references, please see the online version of this paper.