Origin of Cardiac Fibroblasts and the Role of Periostin

Paige Snider, Kara N. Standley, Jian Wang, Mohamad Azhar, Thomas Doetschman, Simon J. Conway

Abstract: Cardiac fibroblasts are the most populous nonmyocyte cell type within the mature heart and are required for extracellular matrix synthesis and deposition, generation of the cardiac skeleton, and to electrically insulate the atria from the ventricles. Significantly, cardiac fibroblasts have also been shown to play an important role in cardiomyocyte growth and expansion of the ventricular chambers during heart development. Although there are currently no cardiac fibroblast-restricted molecular markers, it is generally envisaged that the majority of the cardiac fibroblasts are derived from the proepicardium via epithelial-to-mesenchymal transformation. However, still relatively little is known about when and where the cardiac fibroblasts cells are generated, the lineage of each cell, and how cardiac fibroblasts move to reside in their final position throughout all four cardiac chambers. In this review, we summarize the present understanding regarding the function of Periostin, a useful marker of the noncardiomyocyte lineages, and its role during cardiac morphogenesis. Characterization of the cardiac fibroblast lineage and identification of the signals that maintain, expand and regulate their differentiation will be required to improve our understanding of cardiac function in both normal and pathophysiological states. (Circ Res. 2009; 105:934-947.)

Key Words: heart • cardiac fibroblast • extracellular matrix • Periostin • transforming growth factor-β signaling

Although noncardiomyocytes constitute the majority of the cell types present in the postnatal heart and form the cardiac skeleton within which the cardiac myocytes reside, relatively little is known about how the cardiac interstitial microenvironment is formed and the source of the cardiac fibroblast (CF) lineage. The signals that trigger a secretory fibroblast phenotype and collagen formation (fibrogenesis) as well as the morphogenesis of the CF lineage are also not well understood. The CF is the most abundant noncardiomyocyte cell type present within the postnatal mature heart and is chiefly responsible for deposition of the extracellular matrix (ECM). The ECM is considered a dynamic modulatory network because of the continuous changes in secretory activity which alters both cell environment and response throughout development. Indeed, far from inert, the ECM is characterized by constant reorganization in response to endogenous and exogenous stimuli. The ECM also provides structural support for cardiac myocytes and formation of the elaborate cardiac skeleton. The cardiac skeleton encodes the 3D structure of the heart and is composed of a tough sheet of fibrous connective tissue that electrically isolates the atria from the ventricles, contains all four valves and valvular anchorage tissues, and serves as an attachment site for cardiac muscle fibers.

Balanced synthesis and degradation of this ECM is key to normal cardiovascular development, physiological growth...
of cardiac muscle (exercise), pathological responses to injury (myocardial infarction, hypertrophy, hypertension and ischemia-reperfusion), and for optimal heart function. CFs themselves are a source of paracrine growth factors but can also respond to hormones, growth factors, cytokines and mechanical forces. Expression of receptors for ECM allow CFs to couple mechanical, electric, and sympathetic stimuli to functional responses. Excess transforming growth factor (TGF) deposition of cardiac ECM, resulting in fibrosis, has been associated with activation of various signal transduction pathways in utero, postnataally and during pathophysiological heart overload. These TGF activated CFs are reclassified as “myofibroblasts” because of their unusual morphology, which is characterized by some features of smooth muscle differentiation (can express actin and/or myosin), and functional characteristics. Cardiac fibrosis, which results in stiffening of the ventricular walls, diminished contractility, and abnormalities in cardiac conductance, is a common consequence of heart disease; thus, understanding the role of CFs in sensing, integrating, and responding to stimuli is of both scientific and clinical significance. The role of CFs in pathological remodeling and heart failure has been extensively reviewed elsewhere. Periostin (gene Postn), a TGF superfamily-responsive matricellular protein, has recently emerged as important for collagen fibrillogenesis and overall organization of ECM. In this review we summarize the present knowledge regarding Periostin function within CFs and cardiac morphogenesis.

What Is a Fibroblast?

This is a vague but convenient term for an ill-defined connective tissue cell derived from the primitive mesoderm. Originally, fibroblasts were described in the late 19th century based solely on their location and morphological criteria. Fibroblasts are typically identified by their spindle-shaped flattened morphology, ability to adhere to culture plates, and absence of markers of epithelial, smooth muscle, endothelial, perineural, and histiocytic cells. Fibroblasts synthesize most of the ECM of connective tissue (composed of fibrillar collagens and fibronectin). Their nuclei are large and euchromatic and possess prominent nucleoli. Fibroblasts are characterized as being nonvascular, nonepithelial, and noninflammatory cells. In all tissues, fibroblasts are usually adherent to the fibers which they themselves lay down and thus can form a 3D-network and become embedded within the fibrillar ECM.

Fibroblasts serve diverse vital functions during embryonic development including synthesis of ECM, instructive epithelial differentiation, inflammation regulation, and wound healing. However, the lack of a reliable and specific fibroblast marker is a major limiting factor in the study of fibroblasts in vivo and is assuredly why they remain so poorly understood in both molecular and cellular terms. Although there are several established indicators of fibroblast phenotype, none is exclusive to fibroblasts or are present in all fibroblasts. Fibroblasts are heterogeneous and exhibit topographical differentiation, meaning fibroblasts from different anatomic sites have distinct characteristics and phenotypes which can subsequently be maintained in vitro when fibroblasts are isolated from their surrounding environment and the influence of other cells. Fibroblasts retain a memory of the number of divisions they have completed, and even if suspended from division by being frozen, they will complete only the remainder of their divisions before arresting. Additionally, fibroblasts from differing anatomic sites have distinct transcriptional patterns. Of particular interest, ECM gene expression patterns can vary based on location of fibroblast harvest, as well as genes involved in lipid metabolism, cell signaling pathways that control proliferation, cell migration, and cell fate determination. Similar to local differentiation of skin fibroblasts, atrial fibroblasts are known to express singular gene expression patterns and exhibit different morphology when compared to ventricular fibroblasts. These data correlate with studies showing atrial fibrosis is more severe than ventricular fibrosis in congestive heart failure. These chamber-specific phenotypic differences may arise from the different physiological environments that exist in the atria, which are absent within the ventricles, where the CFs originate from or even when they colonize the various chambers of the heart. Accordingly, it remains unclear whether all CFs are secretory, whether they have multiple origins, and whether they have memory and are prespecified during development of the hearts chambers.

Defining a Cardiac Fibroblasts and Their Distribution Within Developing, Neonatal, and Adult Hearts

The main cellular components of the postnatal heart are cardiac myocytes, endothelial cells, vascular smooth muscle cells (VSMCs), and CFs. Although CFs are the predominant cell type in number, the cardiac myocytes actually occupy the greatest volume. Unlike cardiac myocytes, endothelial cells, and VSMCs, CFs never have a basement membrane and display multiple processes. Thus, CFs can be distinguished from other nonmyocyte lineages via use of laminin or collagen IV to assess absence of a basement membrane in CFs. Additional characteristics include extensive rough endoplasmic reticulum, prominent Golgi apparatus, and abundant cytoplasmic granular material. CFs are found throughout the heart in a 3D network surrounding myocytes (Figure

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**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>CF</td>
<td>cardiac fibroblast</td>
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<tr>
<td>Ddr</td>
<td>Discoidin domain receptor</td>
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<td>E</td>
<td>embryonic day</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>EPDC</td>
<td>epicardially derived cell</td>
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<td>EMT</td>
<td>epithelial-to-mesenchymal transformation</td>
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<td>MHC</td>
<td>myosin heavy chain</td>
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<td>Postn</td>
<td>Periostin</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<td>VIC</td>
<td>valvular interstitial cell</td>
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<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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1) and bridging the gaps between myocardial tissues. Myocytes are arranged in laminae bounded by endomyosial collagen, and the CFs lie within this endomyosial network. Although there are no fibroblast-restricted or even CF-restricted molecular markers, both in utero and mature CFs have been shown to express Discoidin domain receptor (Ddr)2, Vimentin, fibroblast-specific protein (Fsp-1), Periostin (Figure 2), and several key ECM molecules (Table).

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Most studies agree that the first 2 weeks of murine cardiac growth result in at least a doubling of the CF population. However, less consensus is found regarding the relative CF makeup of the adult heart. Early studies on adult rat left ventricle estimated that 65% to 70% of the cells were noncardiomyocytes, whereas recent studies analyzing the total mouse heart by FACS and confocal microscopy estimate a much higher number of cardiomoycytes ≈56% and a ≈44% nonmyocyte content, with only 27% of those staining positive for Ddr2. The densest population of CFs in healthy adult hearts is found around the sinoatrial node, thus providing complete electric insulation. It remains unclear whether CFs are evenly distributed throughout the developing heart and whether they emerge via a uniform or clustered spatiotemporal manner, because quantitative studies describing embryological populations in specific regions of the developing heart have yet to be reported. Intriguingly, confocal staining with Ddr2 in E16 mouse hearts shows localized epicardial surface, atrial, and incomplete ventricular free wall and septal expression. In most species the cardiac system continues to develop through prenatal and postnatal life, but there are wide interspecies differences in timing and duration of specific events, different electrophysiological properties, and distinct TGFβ ligand requirements.

The Many Functions of Cardiac Fibroblasts

It has been estimated that every cardiomyocyte is in direct contact with one or more fibroblasts (Figure 1). Thus, CFs are perfectly positioned to be able to contribute to the structural, biochemical, mechanical, and electric properties of the working myocardium. The primary function of CFs is the synthesis and maintenance of a 3D scaffold for cardiomyocytes which insures the functional integrity of the myocardium. This mechanical scaffold integrates the contrac-
tile activity of individual cells to coordinate the pump function of the heart.\textsuperscript{40,58} In addition to providing a scaffold, the CF fashioned ECM serves to connect cellular structures, distributes mechanical forces throughout the myocardium, and transmits signals via cell surface ECM receptors, and its density regulates fluid movement within the extracardiac environment.\textsuperscript{37} In vitro, interstitial flow has also been shown to regulate TGF\textbeta{} expression, myofibroblast differentiation and ECM alignment in a TGF\textbeta{}-dependent process.\textsuperscript{59} In addition to its primary structural role, the ECM can also play an instructive role, by acting as a repository of growth factors, addition to its primary structural role, the ECM can also play a regulatory role in the development and function of cardiac fibroblasts (CFs) and the myocardium.\textsuperscript{43,60,61}

**Figure 2.** Comparison of known CF markers with Periostin immunolocalization. A, Epicardin\textsuperscript{145} is expressed in epicardial cells surrounding the E15 fetal heart and is also detectable in CFs within the ventricular septum and predominantly the left ventricle (lv). B, In adjacent section to A, Periostin is robustly expressed within the epicardin-expressing lineages, as well as CFs of the right ventricle (rv) and the valvular apparatus. C, Ddr2\textsuperscript{37} is expressed in a number of E15 CFs, particularly surrounding the valves. D, In adjacent section to C, Periostin is expressed within Ddr2-expressing CFs, as well as other CFs and the entire cardiac skeleton. Scale bars: 100 \( \mu{}\text{m} \) (B); 20 \( \mu{}\text{m} \) (D).

With adult CFs,\textsuperscript{44} in addition to stimulating myocyte growth, CFs also have the ability to sense mechanical stress through multiple pathways, including integrins, ion channels, and second messenger responders.\textsuperscript{32} Mechanical stimulation of cultured CFs results in ECM gene expression, growth factor production, and collagenase activity.\textsuperscript{60} CFs have clearly been shown to affect myocardial development and remodeling through direct contact with cardiomyocytes, but their ability to alter cardiomyocyte behavior through noncontact, profibrotic signals is less well understood.\textsuperscript{62}

CFs are connected to one another via specific cadherins and connexins (connexin 40), to the ECM via integrins, and to the myocytes by connexins (connexin 45).\textsuperscript{41,47} In addition to their role in forming insulating barriers, studies have suggested CFs may electrically couple to cardiac myocytes. Through electronic interactions, CFs may synchronize and possibly relay electric activity of multicellular cardiac tissue over distances up to 300 \( \mu{}\text{m} \).\textsuperscript{47,56} Therefore, it is possible that CFs (particularly myofibroblasts) could provide bridges that connect regions of myocytes that otherwise would be electrically isolated by connective tissue. Even though most of the data are from in vitro work, CFs have been shown to synchronize contraction among individual cardiomyocytes, with accompanying membrane potential fluctuations.\textsuperscript{56} Although they do not respond to electric stimulus with the generation of an action potential (nonexcitable), CFs do contribute to myocardial electrophysiology.\textsuperscript{32,63} Coupling of myocytes and CFs would lead to significant changes in action potential duration and upstroke velocity.\textsuperscript{63} When CFs are electrically coupled to myocytes, they could act as current sinks and consequently decrease conduction velocity and maximum depolarization rate of the action potential.\textsuperscript{58,64} Gap junctional communication between CFs and myocytes is thought to be established for short range interaction at the single cell level.\textsuperscript{56} However, the strength of the coupling, how widespread it may be in vivo, and its potential impact on action potential characteristics remain unknown.

**Origin of Cardiac Fibroblasts**

Despite the identification of fibroblasts in the late 19th century and the advent of elegant lineage mapping tools such as Dil labeling, quail chick chimeras, zebrafish photoactivatable (caged) fluorescein marking, and murine loxP/Cre recombinase-mediated genetic cell-marking techniques; still, relatively little is known about the origin and development of the CFs. Fundamental to understanding organogenesis is the ability to determine when and where specific cell types are generated, the ancestry of each cell, and how cells move to reside in their final position. The mesenchymal cells that give rise to the CFs are believed to be derived principally from the embryonic epicardium.\textsuperscript{65–67} However, other sources such as during in utero endocardial cushion epithelial-to-mesenchymal transformation (EMT) and valve morphogenesis\textsuperscript{68} and via the postnatal recruitment of circulating bone marrow cells of hematopoietic origin\textsuperscript{28} has been proposed. To date, lineage mapping in developing mouse and chick embryos has not revealed any significant contribution via the neural crest,\textsuperscript{53,69} second heart field,\textsuperscript{70,71} or endothelial lineages.\textsuperscript{72} Although lineage tracing experiments using endotheli-
al-specific genes such as Tie2-Cre,73 Flk1-Cre,74 and vascular epithelium cadherin75 do not show significant contribution to the in utero mouse cardiac fibroblast lineage, novel data have emerged that show CFs can be derived from endothelial-to-mesenchymal transition in damaged tissues.40,76 These pathological CFs appear to be key for pathogenesis of fibrosis and the facilitation of tumor progression. Because of the present lack of a robust CF-specific molecular marker, the late arrival of CFs after the majority of heart morphogenesis is completed, the difficulty in discriminating between de novo
expression of the various transgenic drivers and when derivative cells are labeled,73 as well as the inherent variability observed among the various lineage mapping tools; it is still conceivable that there may be multiple spatiotemporal sources for subpopulations of the pleiotropic CF lineage. Nevertheless, epicardially derived cells (EPDCs) are currently considered to be the major source of CFs.

The epicardium is the last layer of the vertebrate heart to form, and originates from the transient proepicardium.77 The proepicardium consists of an accumulation of finger-like vesicular protrusions of the pericardial coelomic mesothelium that forms close to the venous pole of the embryonic heart. As a result of proepicardial EMT, the proepicardial ECM becomes populated with mesenchymal cells that migrate and cover the embryonic heart to form the epicardium (reviewed elsewhere77). Following epicardial EMT, the EPDCs are thought to give rise to the majority of interstitial CFs, undifferentiated subepicardial mesenchyme, coronary endotheium and coronary VSMCs. EPDCs can give rise to CFs, either through EMT from the ventricular surface78–81 or by invading the ventricular and atrial walls and migration via the fibrous annulus.80 Epicardial cells additionally play important modulatory roles in myocardial development,81,82 and some controversial data suggest that EPDCs might even differentiate into myocardial cells.83,84 However, this theory has recently been challenged,85 and illustrates the limitations of relying solely on lineage mapping without robust lineage-restricted molecular markers and clear-cut morphological identification criteria.

Experimental studies in both chick and mouse have proved that EPDCs have important roles in heart development.67 The major consequence of the absence of epicardium is a thin myocardial compact layer that results in poor cardiac function and usually leads to in utero lethality.86 In addition, there are often significant abnormalities within cardiac looping, septation, and coronary morphogenesis associated with EPDC dysfunction/ablation.87,88 Given that a subpopulation of EPDCs can differentiate into a variety of different cell types (including coronary endothelium, coronary VSMCs, interstitial cardiac fibroblasts, and atroventricular cushion mesenchymal cells), the EPDCs have even been called the ultimate ‘cardiac stem cell’.67,77,89 When EMT is blocked via antisense targeting of the Ets1/2 transcription factors, epicardial organization is disturbed, and there is a lack of epicardial mesenchyme, coronary VSMCs, and normal myocardial morphology.90 In this experiment, the proepicardium formed normally, and the epicardial cells were able to migrate and cover the cardiac surface, but because of a loss of EMT, the formation of the subepicardial mesenchymal was hindered.90 Both the Wilms’ Tumor gene and erythropoietin growth factor are highly expressed in the epicardium and knockouts of both these genes result in ventricular hypoplasia, pericardial bleeding, and midgestation lethality.91,92 Finally, it has been shown that there is crosstalk between the epicardium and myocardium, and this interaction is essential for normal cardiac development.93–97 Vascular cell adhesion molecule-1 is a surface protein that mediates adhesion via α4 Integrin.94,98,99 Vascular cell adhesion molecule-1 is expressed in the myocardium, whereas α5 is expressed in the epicardium.94,95,100 Both gene knockout mutants have early placental defects in ∼50% of the null embryos, whereas the other half have an absent epicardium and embryonic lethal heart defects.94,95 Another essential interaction is between Gata4 and its cofactor, Fog2 (friend of Gata2).96,97 The transcriptional activity of Gata4 is modulated through a physical interaction with the transcription factor Fog2.96 Targeted disruption shows that Fog2-null embryos die midgestation. Although Fog2 nulls form an intact epicardial layer that properly expresses epicardium-specific genes, EMT is disrupted and the vascular network in the myocardium never forms.96 Significantly, Fog2 expression in the myocardium under the α-myosin heavy chain (αMHC) promoter can rescue coronary vasculature.96 This transgenic rescue experiment is consistent with the idea that signals from the myocardium control epicardial EMT.79,93,96,99,101 Thus, there are several lines of evidence that collectively indicate that when either the epicardium is absent/abnormal or when myocardial crosstalk is compromised, there is a resultant thin myocardial compact layer or “noncompaction of ventricular myocardium” congenital heart defects.102 Thus, signaling between the epicardium, cardiomyocytes and EMT-derived CFs is multi-

![Figure 3. Periostin expression during heart development. A. Postn expression analysis in E13.0 sagittally sectioned embryo hearts. Postn continues to be robustly expressed within both the outflow tract (OFT) and atrioventricular (av) cushions, and its expression in the ventricles increases coincident with increasing CF numbers in the fetal heart (indicated by *). B, Four-chamber view of E13.0 heart, illustrating localization of Postn-expressing cells either side of the interventricular septum (* in B). C, Note punctate Periostin expression throughout the neonatal ventricles and increased Postn expression throughout the atrial fibroblast lineage. D, Immunohistochemistry reveals Periostin is expressed in E12.5 hearts within endocardial cushions/future valves and exhibits punctate expression in CFs and in the epicardium covering the heart. Note robust staining in nonmyocardial cells around the primary interventricular foramen. E, Periostin is localized within CFs in both the E14.0 trabecular (tr) and compact zones (comp), as well as the epicardium (epi). la indicates left atria; lv, liver; lv, left ventricle; r, ribs; ra, right atria; rv, right ventricle; t, thymus. Scale bar=10 μm (E).](image-url)
directional and probably alters as the developing heart undergoes morphogenesis. Although it is presently unclear whether a thin myocardial layer is incapable of compaction or even if CF morphogenesis is affected in the various noncompaction and thin myocardial heart phenotypes, it is interesting to note that primitive zebrafish and newt hearts contain few CFs (mainly confined to the subepicardial layer) and have a spongy (not compact) myocardium with an absence of coronary vessels.\textsuperscript{103}

In addition to the interstitial CFs of the myocardium, the valvular interstitial cells (VICs) are also classed as fibroblasts. However, VICs are different from CFs of the cardiac skeleton/fibrous annulus and interstitial CFs as they are largely thought to be derived from endothelial cells that have undergone EMT.\textsuperscript{73,104} VICs are considered “fibroblast-like” but, unlike CFs, may vary their phenotype in response to ECM, mechanical force, and soluble factors in their microenvironment.\textsuperscript{105} Morphologically and functionally, VICs have characteristics of both CFs and smooth muscle cells.\textsuperscript{106} During development, VICs maintain normal valve structure by producing, secreting, and degrading the ECM in which they are embedded.\textsuperscript{107} Maintenance of ECM architecture provides the mechanical characteristics essential for perpetuating the unique behavior of the valve.\textsuperscript{108}

**Periostin: Functional and Pathological Roles**

Periostin is a 90-kDa secreted protein involved in cell adhesion and contains 4 repetitive fasciclin domains that are similar in sequence to the insect protein fasciclin-1, which is involved in neuronal cell–cell adhesions.\textsuperscript{109} Periostin is expressed in the periosteeum and periodontal ligament,\textsuperscript{110} injured vessels,\textsuperscript{111} metastatic cancer cells,\textsuperscript{112} and in cells undergoing EMT.\textsuperscript{112,113} Although the majority of Periostin made by cells is secreted and deposited extracellularly,\textsuperscript{26} some intracellular staining has been observed but is limited to the secretory apparatus.\textsuperscript{26} As a secreted ECM protein that associates with areas of normal fibrogenesis or pathological fibrosis, Periostin can directly interact with other ECM proteins such as fibronectin, tenasin-C, collagen I/V, and heparin.\textsuperscript{26} Indeed, ultrastructural studies demonstrate that Periostin directly interacts with collagen I and can regulate fibril diameter.\textsuperscript{25} Periostin can also serve as a ligand for select integrins, such as $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$, where it can affect the ability of cancer cells to migrate and/or undergo a mesenchymal transformation.\textsuperscript{112–115} However, it remains unclear whether this ligand-receptor association also occurs during normal heart development.

Periostin is considered a “matricellular protein.” Matricellular proteins are “matrix” proteins that regulate cell function and cell–matrix interactions but do not contribute directly to the physical properties or organization of structures such as fibrils or basal laminae.\textsuperscript{116} Thus, matricellular proteins do not have a direct structural role. In addition to Periostin, the family includes thrombospondin-1, osteonectin, osteopontin, tenasin-C, and tenasin-X.\textsuperscript{117} Matricellular proteins are thought to function via binding matrix proteins and cell surface receptors, as well as modulating expression of cytokines, proteases, and growth factors.\textsuperscript{118} Several mouse models in which matricellular proteins have been knocked out survive embryogenesis and only show mild phenotypes after birth.\textsuperscript{116} Furthermore, the phenotypes of these gene targeted mice are consistent with their minimal contribution to structural integrity and suggest a redundancy during development.

To investigate the requirement of Postn, several groups generated mice that lack Postn.\textsuperscript{24,26,119} To ensure a null allele and facilitate efficient spatiotemporal reporter expression analysis, we replaced the Postn translation start site and first exon with a lacZ reporter gene (Postn\textsuperscript{lacZ}).\textsuperscript{120} Analysis of the endogenous Postn mRNA, protein and Postn\textsuperscript{lacZ} reporter (Figures 2 through 4) spatiotemporal expression patterns in wild types and heterozygotes (in the case of the Postn\textsuperscript{lacZ} reporter), all reveal that Postn is initially detected in E10 to E10.5 CFs, as well as the nascent endocardial cushions. In older E15 hearts, Periostin is robustly expressed in CFs and the epicardial cells that cover the embryonic heart (Figure 2). Within the CF lineage, Periostin is expressed in vivo in all the epicardin- and Ddr2-positive CFs (Figure 2) and in vitro with collagen I and Ddr2.\textsuperscript{26} Periostin is coexpressed intracellularly with collagen I in the cytoplasmic endoplasmic reticulum/Golgci, indicative of active ECM synthesis.\textsuperscript{26} Neonatal and adult CFs continue to robustly express endogenous Postn mRNA, protein and the Postn\textsuperscript{lacZ} reporter (Figures 3 and 4). Thus, the Postn\textsuperscript{lacZ} reporter provides a useful molecular marker of CFs as they colonize the fetal heart and during adult homeostasis (Figure 4). Although Periostin is widely expressed, the majority of the Postn\textsuperscript{lacZ} null mice survived well into adulthood but show smaller overall body weights. Whereas $\approx12\%$ of the nulls die before weaning because of structural valvular anomalies,\textsuperscript{26} the remaining Postn\textsuperscript{lacZ} nulls all develop an early-onset periodontal ligament–like phenotype and craniofacial ECM anomalies.\textsuperscript{120} Analysis of the CF spatiotemporal distribution with Postn-null hearts revealed that CF numbers were unaltered, indicating that Periostin is not required for CF formation but may affect CF function. Significantly, when Postn nulls were subjected to pressure overload stimulation and myocardial infarction, they exhibited reduced fibrosis,\textsuperscript{24} which resulted in aneurysm and rupture of the ventricular wall, especially if subjected to changes in hemodynamic pressure or to a heart attack. Conversely, overexpression of Periostin in the heart protected from rupture in infarcted regions.\textsuperscript{24} Studies of Postn-deficient mice also revealed that Periostin can regulate collagen I and viscoelastic properties of connective tissue.\textsuperscript{25} In fact, Postn is significantly upregulated by both mechanical stretch\textsuperscript{121} and profibrotic TGF$\beta$ signaling.\textsuperscript{26,110} Adult hearts injured via myocardial infarction or exhibiting myocarditis and calcification, exhibit Periostin upregulation specifically within the CF lineage (Figure 5). Thus, loss of Postn results in ECM alterations that are reflected as structural defects\textsuperscript{26,120} and the quantitative amount of Periostin (and perhaps other ECM components) can alter normal physiological interactions between CFs and myocytes that can in turn affect collagen, fibrosis, and scar mechanics.\textsuperscript{122}

Periostin is reduced in pediatric patients with bicuspid aortic valve,\textsuperscript{26} but Periostin levels were increased\textsuperscript{123} in Marfan syndrome patients with thickened heart valves and primary myocardial dysfunction.\textsuperscript{124} In diseased pediatric cardiac valves, Periostin was largely absent from regions
where ECM stratification was lost, collagen fibrils were disorganized, and elastin content reduced. In mouse models of Marfan syndrome (fibrillin-1 mutations), there was loss of tissue integrity attributable to dysfunctional microfibril assembly and function but an elevation in TGFβ superfamily signaling. Significantly, in adult mice hearts, Periostin is strongly upregulated before and during remodeling resulting from long-term pressure overload stimulation and myocardial infarction. These studies demonstrated that levels of Periostin correlated with the amount of collagen produced within the adult heart, and that Periostin expression is restricted to the noncardiomyocyte lineage. Previous controversial studies indicated that Periostin might also be expressed by cardiac myocytes and may directly mediate cardiomyocyte proliferation. However, these studies were equivocal as in vitro studies using neonatal myocytes are rarely free of fibroblasts. Moreover, these studies used dedifferentiating adult rat myocytes cultured for 9 days and a recombinant truncated form of Periostin to ascertain their biological effects. Nevertheless, overexpression of Postn in the mouse heart results in the proper accumulation of Periostin protein in the ECM without detectable intracellular myocyte retention. Furthermore, Postn overexpression did not increase cardiac myocyte number at baseline, nor did it augment incidence associated with cardiac repair after myocardial infarction injury. Similarly, more recent studies have unequivocally demonstrated that Periostin expression is restricted to the noncardiomyocyte lineage.

**Figure 4.** Postn-lacZ knock in reporter expression analysis. A and B, Whole mount lacZ staining of Postn-lacZ E13 (A) and newborn (B) heterozygous mouse hearts reveals robust Postn-lacZ in E13 outflow tract (OFT) cushions, as well as punctate lacZ expression (blue) throughout the left and right ventricles (A). Similarly, Postn-lacZ is expressed throughout the newborn heart ventricles and atria (B), but lacZ is particularly evident within the newborn right atria (ra) when compared to the left atria (la). C through F, Histology reveals Postn-lacZ is confined to aortic, pulmonary, and mitral and tricuspid valve leaflets and the CFs but is absent from cardiomyocytes. Postn-lacZ is strongly expressed in the valve leaflets, the noncardiomyocyte containing annulus (arrow in C) that anchors the valves to the adjacent working myocardium and the neighboring CFs. D, Low-power view of the interatrial septum (as), right superior caval vein (rscv), and the outflow aorta (ao) and pulmonary (p) trunks. Note extensive Postn-lacZ localization lining the superior and inferior interatrial septum. Punctate lacZ expression is seen throughout the right (E) and left newborn ventricles and atria (F). G, Schematic illustration of CF topology in E15 mouse hearts. Subepicardial mesenchyme-derived cells following epicardial EMT migrate into the adjacent myocardium. These EPDCs differentiate into various cell types, including CFs. These CFs surround the coronary arteries and contribute to the cardiac skeleton by their presence in the myocardium, subendothelial spaces, and AV cushions. Scale bars: 2 mm (D); 20 μm (E and F).

**Figure 5.** Elevated Periostin in pathological hearts is confined to the CF lineage. A, Elevated Periostin deposition is observed in adult hearts 7 days after myocardial infarction. Immunohistochemistry for Periostin (brown) within the infarct border zone is shown. Note Periostin is localized to infarct fibroblasts (indicated by *) and is absent from adjacent cardiomyocytes (cm). B and C, Analysis of 7-week-old DBA/2 hearts exhibiting myocardial fibrosis and calcification revealed upregulated Periostin expression (brown DAB staining) is confined to the regions of calcification (arrows), restricted to the activated CFs (indicated by *), and is also absent from ventricular and atrial cardiomyocytes. C, von Kossa staining confirms subepicardial mineralization (black) at sites of myocardial injury.
demonstrated that genetic manipulation of mouse Postn expression in the heart does not affect adult myocyte content, cell cycle activity, or cardiac repair. It is likely that the cardiovascular developmental function of Periostin may include facilitating proper organization of the ECM and in affecting cellular trafficking of CF cells through an injured or reorganizing area. Whereas Ddr2 can act as a collagen receptor, Periostin itself may act as a scaffold-binding protein that enables collagen realignment in response to TGFβ, increasing cross-linking and ensuring normal fibrillogenesis. There are currently no data to support a role for Periostin in stimulating ECM secretion; rather, its absence may result in an ECM that does not sustain a reorganized laminar structure. Indeed, collagen fibrils from Postn-null mice are reduced in size, somewhat disorganized, and less efficiently cross-linked. The close association of Periostin and collagen I, which is among the most abundant ECM proteins in the cardiac skeleton that provides tensile strength, suggests that Periostin may play a role in CF stretch-sensitive signaling and absorption of mechanical stresses. This is born out by the finding that pressure overloading Postn-null hearts exhibit rupture of the ventricular wall, but Postn overexpression protects against rupture.

Similarly, Postn nulls exhibit a periodontal ligament–like phenotype that can be ameliorated via removal of masti- 
catory forces. Finally, Periostin (in conjunction with serum response factors) may act as nodal switch that tips the balance of cardiac skeleton differentiation from a fibroblastic to a myocardial/smooth muscle phenotype, because adult Postn-null epicardial cells can ectopically express myocardial markers. This suggests that secreted Periostin may act at several levels during cardiovascular morphogenesis and postnatal cardiac homeostasis. Thus, Periostin provides a useful molecular marker of the CF population and future characteriza-

tion of the Postn promoter elements may generate valuable lineage mapping/conditional targeting reagents.

**Periostin and TGFβ Relationship**

Notwithstanding the complex and intriguing correlation of deregulated Postn expression levels in both normal and pathological hearts, very little is known about how Periostin is regulated. In addition to the in vivo and in vitro Postn-null data described above, several studies have suggested a link between Periostin and elevated TGFβ signaling. Exogenous addition of TGFβ1 to isolated CFs and cardiomyocytes results in a significant upregulation of Periostin expression within only the CF lineage. Coupled with in vivo analysis of wild-type and Postn-null hearts, these in vitro approaches demonstrate that it is uniquely the CF lineage that expresses Periostin and that Periostin is responsive to TGFβ activation. Furthermore, not only is Periostin directly induced via TGFβ signaling, but Postn itself may be required for normal TGFβ responsiveness. Using isolated E14 PostnLacZ-null and wild-type embryonic fibroblasts within a 3D collagen lattice formation assay system, it was shown that PostnLacZ-null embryonic fibroblasts exhibited reduced 3D lattice formation and TGFβ responsiveness was blunted. To directly test in vivo TGFβ-responsiveness in mature hearts, Periostin expression was examined using cardiac-restricted αMHC-TGFβ1 constitutively active mice. Significantly, TGFβ-induced Periostin upregulation occurs, even in the absence of fibrosis. Inversely, when TGFβ activity is abrogated using an inducible dominant-negative TGFβ type II receptor or TGFβ-neutralizing antibodies, expression of Periostin is reduced. Although the mechanism causing this response is presently unknown, this suggests Periostin deposition may be required to enhance the fibrotic remodeling effects of TGFβ and stabilize microfibril networks within the cardiac skeleton.

Targeted deletions of each individual TGFβ ligand reveals both distinctive and overlapping functions. Thre
mice are largely unaffected and in a postnatal bone and hair follicle maldevelopment. Only Tgf/β2 nulls are also in utero viable but exhibit cleft palate. Because Postn-null mice exhibit reduced TGFβ activity (ie, pSmad2/3), it is possible that Periostin may also be transcriptionally regulated through 1 or more of the TGFβ ligands. In situ hybridization reveals that only Tgf/β2 mRNA colocalizes with Postn, because Tgf/β1 is restricted to the adjacent endothelial lineage and Tgf/β3 is largely confined to the endocardial cushions/valve and annulus (Figure 6). Significantly, measurement of Postn levels in isolated Tgf/β1 null, β2 nulls, and β3 null Tgf/β1-, -β2, and -β3 null ventricles reveals that Postn is unaffected in Tgf/β1- and -β3-null hearts but is significantly reduced in Tgf/β2-null hearts (Figure 6). Furthermore, Periostin protein expression is also significantly diminished in Tgf/β2-null ventricles (Figure 5). Given that several studies have shown exogenous nonphysiological levels of TGFβ1 and pathological TGFβ1 overexpression can both induce Postn, these data suggest Periostin is responsive to TGFβ1 but requires TGFβ2 for normal spatiotemporal expression within the in utero heart. Given the diminished TGFβ1 responsiveness observed in Postn-null embryonic CFs, it is tempting to speculate that the relative composition of the ECM imparts contextual specificity to Periostin-TGFβ1 signaling by either concentrating the ligands at sites of intended function (positive regulation) or by inhibiting their bioavailability (negative regulation).

Conclusions
The past several years have yielded remarkable insights and progress in identifying and mapping the various cell lineages that initially give rise to the developing heart and deciphering many of the key morphological events that are required for both normal heart development and the underlying causes of congenital heart defects. Despite this recent progress, our understanding of the mechanisms of induction and lineage specification of early noncardiomyocyte cell fate is still rudimentary, and the signals that instruct epicardial precursors to select a CF cell lineage remain unclear. Progress toward understanding the molecular, cellular, and morphological events required to generate the hearts interstitium and the cardiac skeleton that provides its 3D form have been hampered via the lack of suitable molecular markers and appropriate lineage analysis tools. We propose that the matricellular Periostin protein provides a useful system with which to probe the functional role of the CF lineage and noncardiomyocyte heart development.

A challenge now facing investigators is characterization and isolation of the key progenitor cells within the developing embryo that give rise to the CF lineage. The identification of cell surface markers of putative CF progenitor/stem cells would be invaluable for the investigation of the potential stem cell niches in the developing heart. Isolation of these progenitor cells would allow delineation of their molecular profile and would accelerate discoveries of the signals instructing these cells to select cardiovascular cell fates. Understanding the biology of CF progenitor cells as the heart forms would also allow a detailed search of the adult heart to assess whether progenitors of similar phenotype remain in the postnatal heart or may be reactivated during repair after cardiac injury.

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Disclosures
None.

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