Neonatal and Adult Cardiovascular Pathophysiological Remodeling and Repair

Developmental Role of Periostin

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The neonatal heart undergoes normal hypertrophy or compensation to complete development and adapt to increased systolic pressures. Hypertrophy and increased neonatal wall stiffness are associated with a doubling of the number of fibroblasts and *de novo* formation of collagen. Normal postnatal remodeling is completed within 3–4 weeks after birth but can be rekindled in adult life in response to environmental signals that lead to pathological hypertrophy, fibrosis, and heart failure. The signals that trigger fibroblast and collagen formation (fibrosis) as well as the origin and differentiation of the cardiac fibroblast lineage are not well understood. Using mice studies and a single-cell engraftment model, we have shown that cardiac fibroblasts are derived from two extracardiac sources: the embryonic proepicardial organ and the recruitment of circulating bone marrow cells of hematopoietic stem cell origin. Periostin, a matricellular protein, is normally expressed in differentiating fibroblasts but its expression is elevated several fold in pathological remodeling and heart failure. Our hypothesis that periostin is profibrogenic (i.e., it promotes differentiation of progenitor mesenchymal cells into fibroblasts and their secretion and compaction of collagen) was tested using isolated and cultured embryonic, neonatal, and adult wild-type and periostin-null, nonmyocyte populations. Our findings indicate that abrogation of periostin by targeted gene deletion inhibits differentiation of nonmyocyte progenitor cells or permits misdirection into a cardiomyocyte lineage. However, if cultured with periostin or forced to express periostin, they became fibroblasts. Periostin plays a significant role in promoting fibrogenesis residual stress, and tensile testings indicated that periostin played an essential regulatory role in maintaining the biomechanical properties of the adult myocardium. These findings indicate that periostin is a profibrogenic matricellular protein that promotes collagen fibrogenesis, inhibits differentiation of progenitor cells into cardiomyocytes, and is essential for maintaining the biomechanical properties of the adult myocardium.

Key words: **periostin; fasciclin; myocardial remodeling; hypertrophy; fibrosis; cardiac fibroblast; myocyte; myocardial infarction; heart failure**

Introduction

Heart Development Extends beyond Intrauterine Life

The neonatal heart undergoes rapid enlargement and adaptive (or physiological) "remodeling" to complete development and maturation. It is a unique transitional period that bridges the embryonic/fetal period of heart development and the fully defined adult heart. Based on pioneering studies by Borg *et al.*,[1] it is now generally recognized that the neonatal heart adapts (remodels) to sudden increased systolic pressures following birth by increasing ventricular wall thickness and stiffness (i.e., tensile strength). This is a result of a twofold increase in the number of fibroblasts and the formation, compaction, and alignment of collagen fibrils that envelop myocytes as an endomysial-like collagenous network.[2] This organization provides

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for potential "contact-signaling" between myocytes and fibroblasts, the fibroblasts themselves, or cell–extracellular matrix (ECM) contacts that prepare the postnatal myocardial wall to structurally adapt and respond to increases in blood pressure after birth.[3] Thus, the fibroblast is potentially a key player in neonatal development.

In the mouse, the unique, fibroblast-mediated, adaptive/remodeling period progressively diminishes after 1 week and the mature phenotype is fully established by 30 days. However, remodeling can be rekindled in adult life in response to environmental changes (e.g., pressure overload or ischemic injury) that may lead to an increase in fibroblasts and collagen content. These normal adaptive responses may eventually become pathological, resulting in fibrosis and increased wall stiffness.[3,4] Here, we address some questions that relate to the mechanisms of neonatal heart development and their possible relationship to pathological remodeling in an adult heart (e.g., if the myocardium is injured):

1. What is the origin of cardiac fibroblasts? Are they derived from a single population of progenitor cells that are carried over from intrauterine to postnatal and adult life or are new cells added or recruited into the heart?
2. Are there any signals that direct postnatal cardiac progenitor cells into a fibroblast lineage or inhibits their differentiation into other mesodermal lineages (e.g., cardiac muscle, bone, or cartilage)?
3. Are these same signals re-expressed or elevated in the adult heart following acute injuries that result in fibrosis and ventricular dysfunction?

Answers to these questions bear directly upon human heart disease, which remains one of the most prevalent and life-threatening diseases in the world and one of the fastest growing.

The Origin(s) of Cardiac Fibroblasts

Surprisingly, while there is considerable knowledge on the structure and function of neonatal cardiac myocytes, much less is known about the role(s) of fibroblasts during postnatal development or pathological remodeling. One reason is that their developmental origin is unclear. Are we born with all the fibroblasts we will ever have or are they a dynamic cell population that can be expanded or recruited from elsewhere? Information from our embryonic chick and adult mouse studies suggests there are two major sources of cardiac fibroblasts: embryonic proepicardial organ (PEO) and circulating progenitor cells derived from adult bone marrow stem cells (as discussed and shown below and in Ref. 11).

The Proepicardial Organ

The PEO is a sac-like vascular structure that is derived from the coelomic mesothelium located at the venous inlet (sinuatrial) pole of the heart.[5] The PEO gives origin to cells that migrate as an epithelium over the surface of the entire heart to form the embryonic epicardium. The epicardium, in turn, undergoes a cell autonomous epithelial to mesenchymal transformation (EMT) to form epicardial-derived cells (EPDCs) that accumulate as mesenchyme between the epicardium and myocardium. During the late embryonic and fetal period, the EPDCs invade the atrial and ventricular walls and functionally interact with cardiomyocytes to establish a compact myocardium.[6] FIGURE 1 was prepared in collaboration with a colleague, Dr. John Burch (Fox Chase Cancer Center, Philadelphia, PA), to illustrate the invasion of EPDC into the ventricular myocardium as revealed by their expression of enhanced green fluorescent protein (EGFP) driven by the Wilms' tumor promoter, which is expressed in the epicardium and EPDCs (and not other heart cell types).[7] Failure of EPDCs to invade the myocardium results in lethality as a result of the failure to form a compact myocardium, indicating that from the beginning of cardiac embryogenesis, there is an interaction between the fibroblasts (or their progenitors) and cardiomyocytes that is necessary for myocardial growth, survival, and function.[6,8]

While the EPDCs are an immediate progenitor of cardiac fibroblasts, it is important to recognize that they are also multipotential cells. They have potential to differentiate *in vitro* into endothelial cells, smooth muscle, and cardiomyocytes.[5,6] However, *in vivo*, during normal embryonic life, most EPDC within the wall of the heart progressively differentiate into fibroblasts that oscillate or transdifferentiate between two phenotypes: one is spindle shaped; the other is more rounded and sometimes called a *myofibroblast* because it expresses α-smooth muscle actin. Over time, expression of α-smooth muscle actin is suppressed and most EPDC derivatives assume a more spindle-shaped phenotype. After birth and into adult life, the myofibroblast phenotype can be reactivated by transforming growth factor (TGF)β signaling during a pathogenic remodeling event.[9,10]

Recruited Fibrogenic Progenitor Cells

In addition to the EPDC-derived progenitor cells, we have found evidence for postnatal recruitment

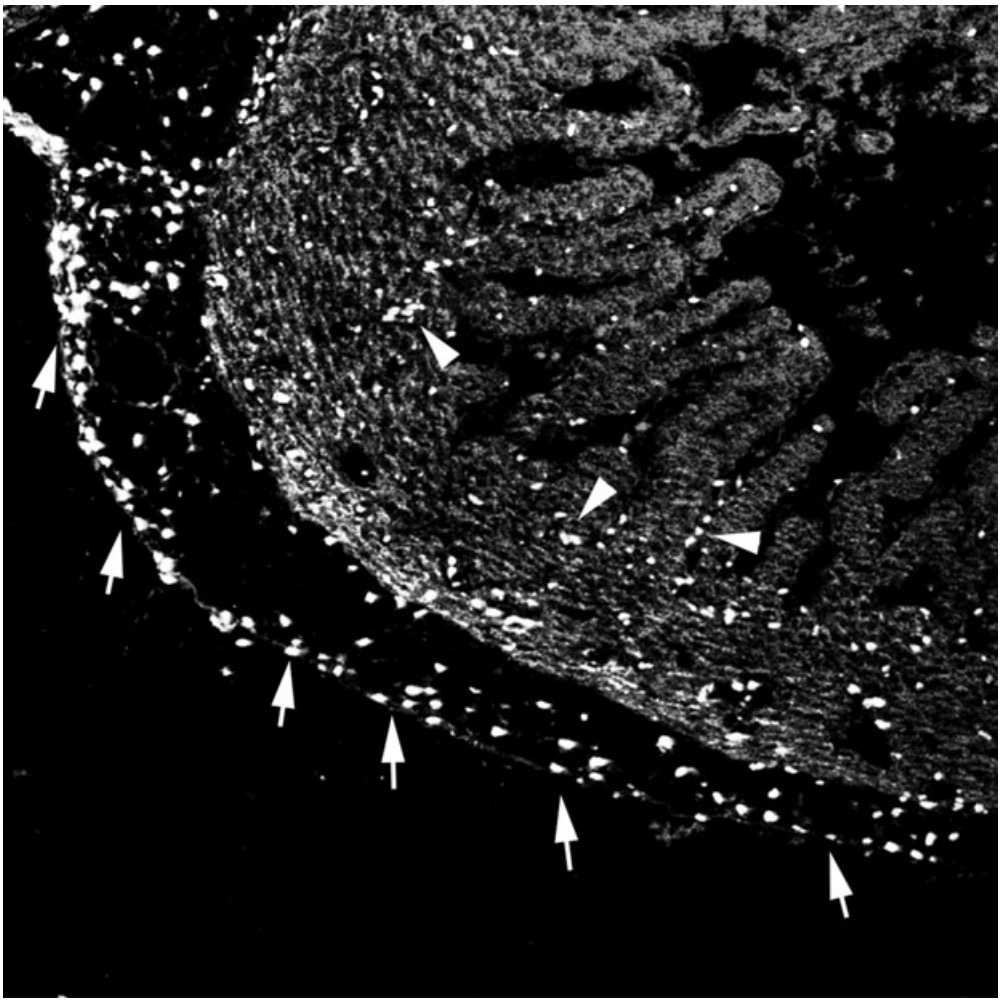

FIGURE 1. Contribution of EPDCs to the ventricular myocardium. Sagittal section of E16.5 wild-type 1 (WT1)-EGFP transgenic mouse heart showing EPDC cells invading the ventricular wall.

of circulating fibroblast progenitor cells into the ventricular myocardium.[11] This can possibly represent a continuation of a recruitment process that has begun before birth or hatching.[12] As described in Visconti *et al.*,[11,13,14] the progeny of a single (EGFP+) donor hematopoietic stem cell (HSC) injected to repopulate the bone marrow of a lethally irradiated adult mouse host will also give rise to EGFP+ cells in the adult heart. Current controversy regarding the lineage potential of any given engrafted stem cell may be because most studies evaluating stem cell potential invariably use mixed cell populations.[15,16] Thus, we believe that the definitive assignment of fate (or lineage) should be based on the analysis of a *single* donor cell's ability to achieve long-term organ engraftment and multilineage hematopoietic reconstitution of bone marrow when injected into the tail vein of an irradiated mouse host. This permits the full potential of a single HSC to be evaluated *in vivo*. Based on our results using this method, we have concluded that circulating bone marrow HSCs engraft into the heart and give rise to fibroblasts and myofibroblasts, which, as noted above, may be the same cell type but reflect different secretory or contractile states because of different epigenetic influences.[11,17]

Whatever their origin (EPDC or HSC), it is important to recognize that undifferentiated fibroblastic progenitor cells are present in normal newborn and adult hearts. They do not express traditional fibroblast markers or only very low levels of the markers unless induced to differentiate into a mature fibroblast. Admittedly, the fibroblastic phenotype is hard

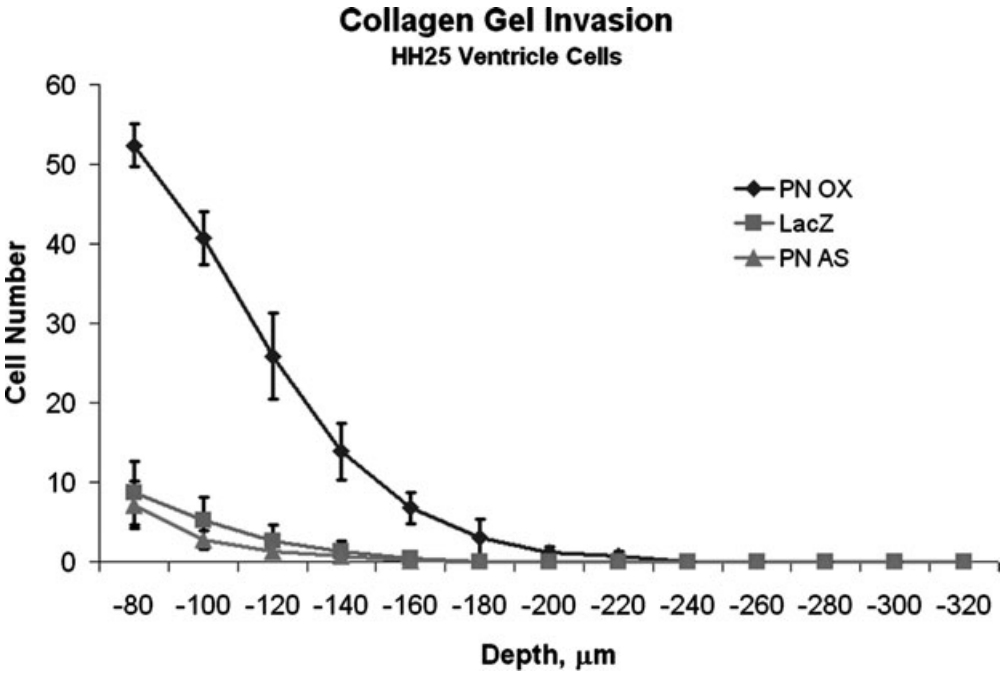

FIGURE 2. Periostin induces myocardial invasion. Ventricular apexes were isolated from HH25 chick hearts and infected with either the periostin-expressing virus (PNOX), an antisense periostin virus (PNAS), or control virus (LacZ) in hanging drop culture. The hanging drop aggregates were placed on top of type I collagen gels (1.5 mg/mL) and cultured for 3 days. The number of invasive cells, at specified depths, were counted. Forced expression of periostin in embryonic myocytes induced invasive migratory behavior not observed in empty vector controls.

to characterize satisfactorily. In part, this is because fibroblasts are heterogeneous. Arbitrarily, we assign a fibroblast phenotype to nonmyocyte cells that strongly express type I collagen (mRNA and protein), discoid domain receptor 2 (DDR2), type 3 procollagen, fibroblast surface antigen (FSA), α-smooth muscle actin, vimentin, and periostin.[4] Using established methods to isolate myocytes versus nonmyocytes,[1] we have found a small population (15%) of neonatal nonmyocytes that do not express fibroblast markers and, if plated on the surface of a collagen gel, do not exhibit invasive migratory behavior. Conversely, nonmyocyte cells that express fibroblast markers, like periostin, do invade the gel lattice. Thus, migratory potential may be a useful tool for determining differentiation status of neonatal or adult nonmyocytes and signals that might promote their expression of fibroblast markers (and not other lineage markers) and their invasive migratory activity. To test this possibility, we explanted embryonic chick ventricular myocytes, which normally do not express periostin or migrate as individual cells, onto a collagen gel lattice and assessed their potential to become invasive if infected with full-length periostin cDNA viral vectors. Forced expression of periostin in embryonic myocytes induced invasive migratory behavior that was not observed in empty vector controls (FIG. 2). Additionally, migrating embryonic myocytes infected with periostin vectors also switched from expression of a myocyte sarcomeric marker protein to a smooth muscle cell marker protein. Collectively, these findings suggest that periostin is a candidate regulator for activating migration and/or modifying differentiation of cardiac progenitor cells.

Signal(s) that Promote Differentiation of Neonatal or Adult Cardiac Progenitor Cells into Fibroblasts

The two major adaptive changes within the left ventricular myocardium during neonatal life are an increase in fibroblast number and the progressive secretion and organization of a collagenous (endomysial-like) network.[18] The mechanism of the increase in fibroblasts is not known but its expression profile and the phenotype of its targeted deletion[19] invoke a regulatory role for periostin. Periostin is one of several fibroblast markers that are initially expressed at low levels in the prevalvular and EPDC mesenchyme of the embryonic heart. Periostin expression peaks after

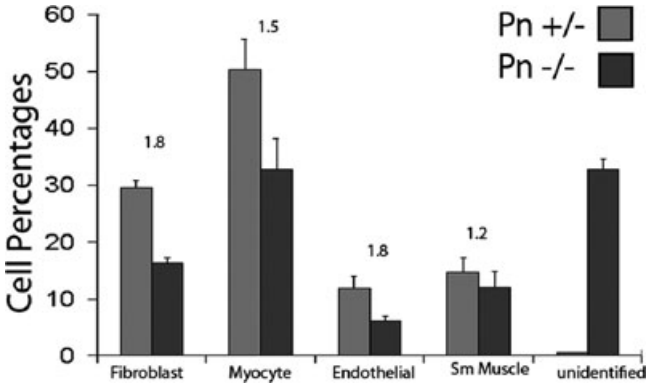

FIGURE 3. FACS analysis of wild-type and periostin-null hearts. Twelve-week-old hearts were prepared for fluorescence-activated cell sorting (FACS) using antibody markers for fibroblasts (DDR2), smooth muscle (α-SMA), myocytes (α-MHC), and endothelial cells (PECAM/CD31). In the periostin −/− mice, a large percentage of unidentified cells exist.

birth early in the neonatal period and then falls to a baseline level that is maintained throughout life; it is not expressed in neonatal or adult cardiomyocytes but only fibroblasts.[3,19,20] Thus, periostin expression correlates with increased formation of fibroblasts and collagen, presumably in response to changes in hemodynamic forces immediately after birth.

What is Periostin?

Periostin is an evolutionary-conserved ECM protein that has four domains: a signal sequence, an N-terminal domain that contains cysteine residues, a carboxyl terminus that can be alternatively spliced, and four coiled fasciclin (fas) domains that have closest homology to the *Drosophila fasciclin 1* gene.[21] In *Drosophila*, the ancestral fasciclin domain functioned as an adhesion molecule linked to axonal guidance, migration, and differentiation.[21–23] Periostin can interact *in vivo* with other ECM scaffold proteins, especially collagens.[24,25] Isoforms lacking the entire carboxyl domain inhibited cell motility and migration.[24] Known receptors for periostin are integrins (α_V/β_3 and α_V/β_5).[26,27] Periostin binding to integrins is thought to be through highly conserved H1 and H2 peptide stretches (but not RGD sequences) present within the fasciclin domain having "YH" and Asp-Ile motifs.[21,27] We have also found that periostin binding to integrins α_V/β_3 and β_1 on cardiac mesenchymal progenitor cells can initiate signaling related to migration and collagen cross-linking or compaction transduced through Rho and phosphoinositide-3 (PI3) kinases.[28] Thus, there is an integrin-based receptor mechanism for mediating the potential effects of periostin on neonatal ventricular remodeling. However, we cannot yet rule out that other receptors may exist for transducing periostin signals.

Periostin as a Matricellular Protein

Bornstein and colleagues[29,30] have proposed that there is a family of functionally related secreted proteins, called *matricellular* proteins. The latter derive their complex functions from their ability to interact with multiple cell surface receptors, especially integrins, cytokines, growth factors, and proteases; but matricellular proteins can also bind directly to structural or scaffold proteins. Examples of matricellular proteins include thrombospondin, tenascin-C, osteopontin, CCN1, and SPARC.[31] The expression of this unique family of ECM proteins is most prominent during development and growth or in response to injury.[32] Based on its known biological roles, we propose that periostin also qualifies as a matricellular protein. As such, we would expect that the contextual nature of its functions include dynamic contact-signaling between mesenchyme progenitor cells, fibroblasts and mesenchyme, or fibroblasts and cardiomyocytes.

Periostin Knockout Mice

To test whether periostin promotes differentiation of cardiac mesenchymal progenitor cells into fibroblasts *in vivo*, two periostin knockout mice were generated.[19,33] In both lines, deletion of periostin inhibited or delayed differentiation of mesenchyme into fibroblasts, which resulted postnatally in loss of fibrous tissue or abnormally differentiated cells (e.g., ectopic cardiomyocytes, bone, or cartilage). At 12 weeks, there remained a large population of undifferentiated mesenchymal-like cells (33%) in periostin-null hearts whose lineage identity could not be determined with the same marker antibodies that were sufficient to identify 100% of the cells isolated from wild-type adult hearts[34] (FIG. 3). Whether

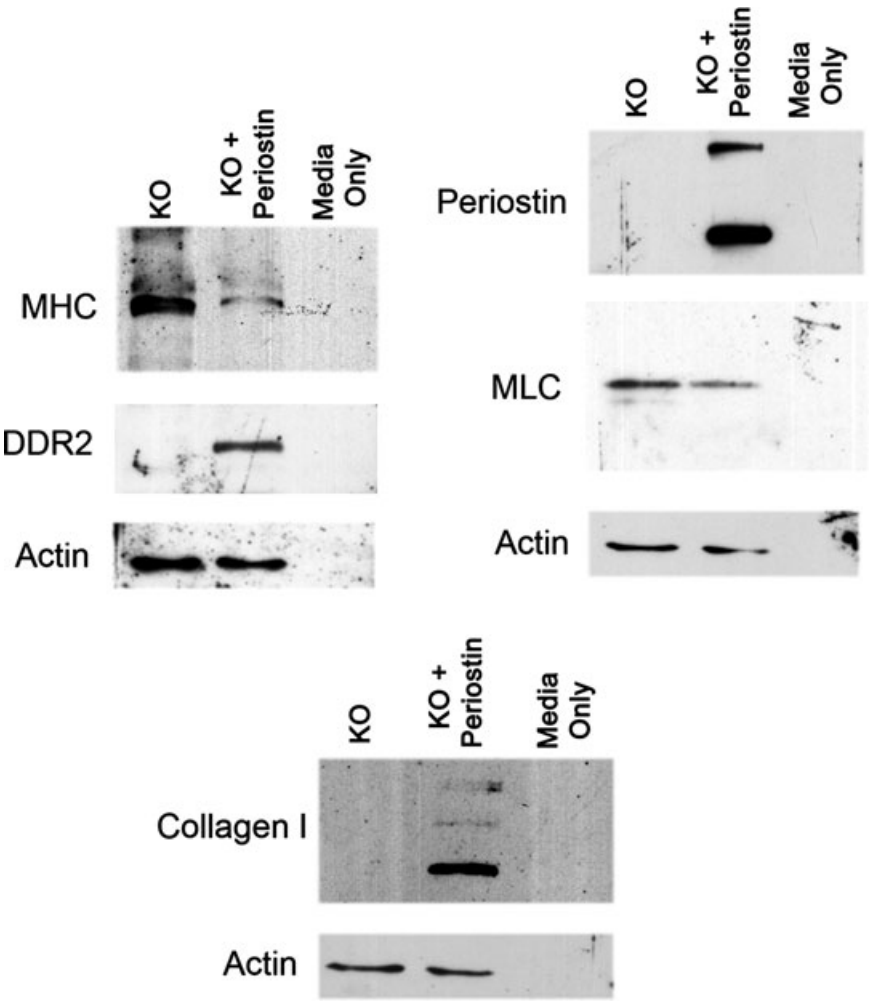

FIGURE 4. Periostin functions as a nodal differentiation switch. Western blot analysis of isolated cardiac mesenchymal cells from periostin-null mice. One group of cells was given the purified periostin protein whereas the other group was not. After 7 days in culture, protein lysates were taken and expression of fibroblasts markers (DDR2, periostin, and collagen I) or myocytes (MHC and MLC) were analyzed. Periostin expression is not only required for the expression of fibroblast markers but also for the repression of myocardial proteins.

these cells came from EPDC or HSC has not been determined, but these genetic findings and those provided by transfecting periostin genes into mesenchymal progenitor cells suggest that periostin is needed to promote full and complete differentiation of cardiac progenitor cells into postnatal fibroblasts.

Periostin as a "Nodal" Differentiation Switch

There is also evidence that periostin may act as a binary differentiation signal that determines if cardiac mesenchymal progenitor cells differentiate into fibroblastic lineages versus other mesodermal phenotypes, especially cardiac muscle. As shown in FIGURE 4, the addition of purified full-length periostin protein to cultures of periostin-null cardiac mesenchymal cells not only "rescued" collagen synthesis and expression of fibroblast markers but also suppressed the expression of myocyte marker proteins. Infarcts created in wild-type versus periostin-null hearts indicated that fibrous scar tissue is reduced in periostin nulls, replaced instead by viable cardiomyocytes.[33] Collectively, these data indicate that periostin is a signaling protein in embryonic and postnatal life that functions to promote differentiation of mesenchymal progenitor cells into fibroblasts while inhibiting their differentiation into cardiomyocytes.

Is There a Developmental Basis for Adult Cardiac Pathological Remodeling?

Remodeling can be a normal developmental process, as in neonatal life in response to environmental changes. However, if the modulating signals continue after recovery or adaptation to a changing environment, the remodeling process can become detrimental to cardiac function and ultimately lead to irreversible damage and heart failure.[35,36] As noted above, a spike in periostin expression occurs during neonatal development, which correlates with increased numbers of fibroblasts and new formation of fibrous ECM components, especially collagen.[1,2,4] While there are numerous signals that can trigger or mediate adult remodeling ranging from extracellular ligands for G protein coupled receptors to receptor tyrosine kinases,[37,38] there are also extensive data suggesting that periostin may be a key trigger. This is based on an eightfold to 40-fold increase in periostin expression when pathological remodeling is induced in adult hearts by acute or chronic injuries.[39,40] Using a TAC banding model to induce chronic pressure overload and cardiac hypertrophy, we found that periostin was upregulated over 64-fold and accompanied by extensive collagen deposition and fibrosis.[19] Katsuragi *et al.*[39,41] reported that elevated expression of periostin correlated with increased fibrosis during ventricular dilation but also noted a loss of ventricular cardiomyocytes, indicating that periostin may not only promote collagen formation and fibrosis in the adult heart but may also affect the fate of adult cardiomyocytes. Various aspects of remodeling of cardiac myocytes have been explored after infarction, hypertrophy, and congestive failure. For example, cardiomyocytes can (i) increase in size (hypertrophy),[37] (ii) undergo apoptosis,[42,43] or (iii) activate an embryonic gene program (e.g., periostin) and/or revert to a myofibroblastic phenotype.[44–46] Whether any of these changes are caused indirectly by periostin promoting formation of collagen fibrils (which, in turn, interact in some way with myocardial cell surfaces) or directly by mediating an inductive interaction between adult fibroblasts and myocytes is an important unresolved question. If embryonic development serves as a relevant precedent, then the answer would seem to be that periostin can directly signal changes in postnatal cardiac myocytes. During development, at sites where embryonic cardiac mesenchyme directly contacts myocytes (e.g., as at the atrioventricular (AV) junctional myocardium), periostin expression is elevated and the fate of the adjacent myocytes is to "disappear" or retract from the original boundary interface.[47–49] In periostin nulls or in other mouse or chick embryos where periostin expression is inhibited, myocardial cells that normally disappear persist, resulting in conduction system disturbances or the failure of AV valve leaflets to delaminate.[19,48,50] As shown in FIGURE 5, when neonatal populations of isolated cardiomyocytes are co-cultured for 48 h with periostin-positive neonatal fibroblasts, there is a progressive disruption of cardiomyocyte cell:cell associations followed by their gradual loss of myosin+ sarcomeric organization. By 72 h, periostin-positive fibroblastic cells fill spaces once occupied by an epithelial monolayer of myocytes. These findings suggest that the contact of neonatal myocytes with fibroblastic cells expressing periostin can directly modify the phenotypic stability of myocytes and possibly their survival.

Material Properties and Elevated Expression of Periostin

Because of its link to fibrosis and pathological remodeling, it is important to know if the passive biomechanical properties of the myocardium are modified as a result of enhancing or decreasing periostin. The small size of mouse hearts limits the number of *in vivo* biomechanical assays possible. Nevertheless, we have been able to perform preliminary "residual stress and tensile testing" studies. Residual stress has been previously shown to be important in the cardiovascular system for distributing loads and reducing peak myocardial wall stress. Decreased residual stress may indicate increased tissue stiffness resulting in impaired diastolic function.[51] The classical residual stress assay is the open angle measurement of the left ventricle.[52] Briefly, we performed this assay on excised wild-type versus periostin-null adult (3 months) hearts. Our results, shown in FIGURE 6, demonstrate that periostin deficiency leads to reduced residual stress. Because collagen matrix is known to have a strongly nonlinear response, we have taken these results to suggest that periostin deficiency leads to impaired collagen production and organization which, in turn, reduces wall stiffness and residual stress important for systolic and diastolic function. This conclusion is strongly supported by Oka *et al*,[33] who created a periostin overexpressor mouse and a periostin knockout mouse and subjected each to increased blood pressures or surgically created myocardial infarcts. Null animals were prone to aneurysm and rupture of the ventricular wall, whereas overexpression of periostin in the heart increased fibrosis and protected from rupture, especially after experimentally inducing a heart attack. This further indicates that a developmental protein, such as periostin, can have important regulatory effects on adaptive

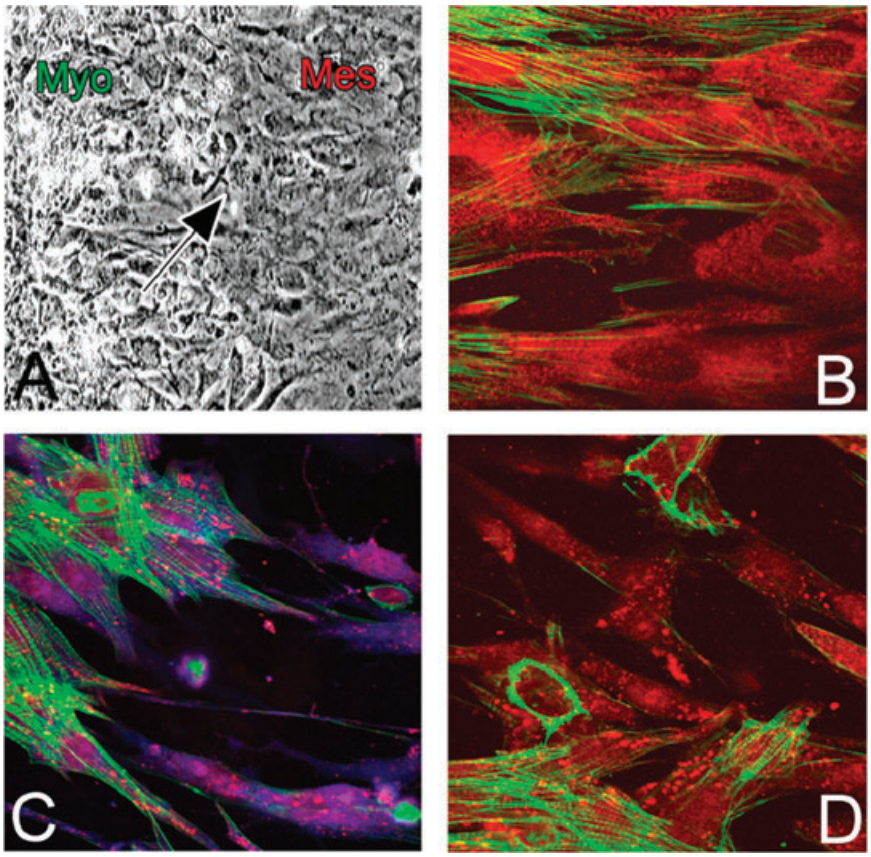

FIGURE 5. Fibroblast–myocyte co-cultures. **(A)** Bright field image of myocyte–fibroblast interaction 24 h after the spacer bar has been removed. *Arrow* points to original location of the spacer bar. **(B)** Periostin staining (red) of fibroblasts that have contacted myocytes (green) after 24 h in culture. **(C, D)** Forty-eight h in culture, myocytes in contact with fibroblasts form migratory-like cell processes coincident with reorganization of their actin cytoskeleton as seen by rings of phalloidin-positive material (green).

responses to increasing blood pressure during neonatal life and can also adjust ventricular wall stiffness in adult life in response to environmental signals.

Remodeling, Repair, and Recruitment

HSCs derived from the bone marrow circulate as cardiac progenitor cells that engraft into the heart as both valvular or ventricular interstitial cells. These engrafted cells strongly express fibroblast markers including periostin.[11,14] The question then becomes does engraftment change when the heart is injured (e.g., by a surgically created infarct or cryoinjury). In response to this question, we now know that (i) periostin is strongly upregulated in the heart following ischemic injury,[33,40] (ii) the number of fibroblasts increase at the site of the infarct,[33] and (iii) fibroblasts of the infarct intensely express periostin.[11] What is not known is (i) whether the fibroblasts of scar tissue are recruited from circulating progenitor cells or derived from proliferation of the endogenous pool of interstitial fibroblasts and (ii) if periostin directs their differentiation into a fibroblast lineage. To answer these questions we have initiated studies using our single-cell engraftment approach in which a single wild-type EGFP+ HSC is injected into the tail vein of an irradiated wild type or host. In preliminary studies, we have found that EGFP+ cells comprise the majority of cells in the infarct that is formed in an irradiated, wild-type host. Gender mismatch controls were run and ruled out cell fusion. *This finding indicates that the fibroblasts are recruited from the HSCs of the bone marrow and thus are the most likely source of the elevated expression of periostin seen in the ischemic injury model*. Finally, when an ischemic injury is created in a periostin-null mouse, the size of the infarct is significantly reduced and many of the cells forming the infarct resemble cardiomyocytes.[33] Thus, abrogation of periostin expression by gene deletion

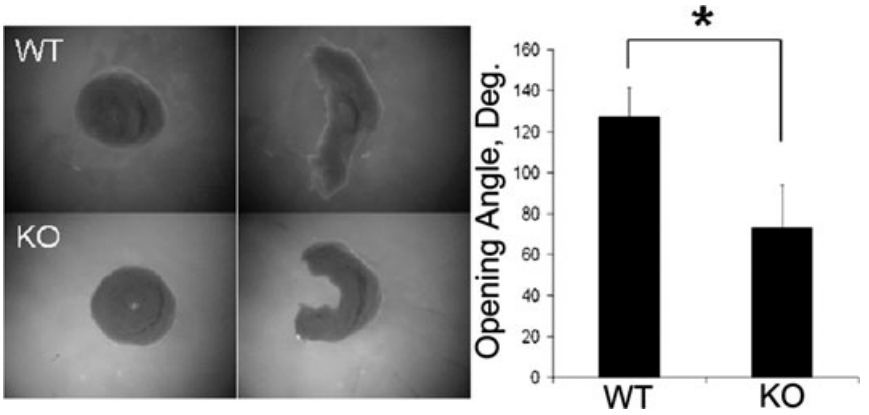

FIGURE 6. Opening angle measurements on wild-type and periostin −/− mice. Adult hearts from wild-type (WT) and periostin −/− (KO) mice were isolated and used for determining opening angle measurements. Periostin −/− mice exhibit a reduction in the opening angle measurement(*) indicating a compromise in myocardial wall stiffness.

results in reduced scar formation and inhibited and modified differentiation of progenitor cells into fibroblasts. It remains to be determined if wild-type HSCs can rescue scar formation and differentiation into fibroblasts in periostin nulls or, conversely, if a single periostin −/−, EGFP+ HSC injected into the bloodstream of a host animal, can modify scar formation and differentiation in a wild-type (irradiated) host.

Conclusions

It is clear that in either normal physiological or pathological remodeling, the usual developmentally expressed proteins, such as periostin, can be important players in the qualitative and quantitative aspects of fibrogenesis and fibrosis. Such proteins could have great potential for controlling the onset and/or progression of myocardial remodeling from normal adaptive responses to pathological changes that alter performance and possibly lead to catastrophic heart failure. Therefore, a selective spatiotemporal reduction of normal periostin expression in the infarct region following a heart attack may provide a plausible therapeutic intervention that would result in improved myocardial remodeling and overall long-term enhancement of cardiac function.

Acknowledgments

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Conflicts of Interest

The authors declare no conflicts of interest.

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