Periostin Is Required for Maturation and Extracellular Matrix Stabilization of Noncardiomyocyte Lineages of the Heart

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Abstract—The secreted periostin protein, which marks mesenchymal cells in endocardial cushions following epithelial–mesenchymal transformation and in mature valves following remodeling, is a putative valvulogenesis target molecule. Indeed, periostin is expressed throughout cardiovascular morphogenesis and in all 4 adult mice valves (annulus and leaflets). Additionally, periostin is expressed throughout the fibrous cardiac skeleton and endocardial cushions in the developing heart but is absent from both normal and/or pathological mouse cardiomyocytes. Periostin (perI^{lox/lo}C) knockout mice exhibit viable valve disease, with neonatal lethality in a minority and latent disease with leaflet abnormalities in the viable majority. Surviving perI^{lox/lo}C null leaflets are truncated, contain ectopic cardiomyocytes and smooth muscle, misexpress the cartilage proteoglycan aggrecan, demonstrate disorganized matrix stratification, and exhibit reduced transforming growth factor-β signaling. Neonatal perI^{lox/lo}C nulls that die (14%) display additional defects, including leaflet discontinuities, delamination defects, and deposition of acellular extracellular matrix. Assessment of collagen production, 3D lattice formation ability, and transforming growth factor-β responsiveness indicate periostin-deficient fibroblasts are unable to support normal valvular remodeling and establishment of a mature cardiac skeleton. Furthermore, pediatric stenotic bicuspid aortic valves that have lost normal extracellular matrix trilaminar stratification have greatly reduced periostin. This suggests that loss of periostin results in inappropriate differentiation of mesenchymal cushion cells and valvular abnormalities via a transforming growth factor-β-dependent pathway during establishment of the mature heart. Thus, perI^{lox/lo}C knockouts provide a new model of viable latent valve disease. (Circ Res. 2008;102:752-760.)

Key Words: heart development ■ periostin ■ cardiac skeleton ■ valve ■ mouse

Defects within valves and associated fibrous structures of the heart are the most common congenital defect subtype, accounting for 25% to 30% of all malformations.1 Despite epidemiology studies showing an increased incidence of valve disease,2 little is known about its pathogenesis. Recent evidence implicates genetic causes and dysregulation of genes important during valvular development.3–7 Although some congenital valvular defects are detected at birth, the majority only become apparent later in life and increase the risk of subsequent morbidity and mortality.1,4

During heart development, cushion mesenchyme is repositioned and remodeled to form projections that give rise to stress-resistant valves, whereas atrioventricular (AV) mesenchyme gives rise to the annulus that provides anchorage for the mitral and tricuspid leaflets to the working myocardium. Similarly, nonvalvular outflow tract (OFT) mesenchymatous cells populate the fibrous aortic and pulmonary attachment rings. The structural organization of the collagogenous extracellular matrix (ECM) network is essential for cardiac function,8 and the cardiac fibroblast is the principal cell type responsible for producing components of the ECM.9 Although much is known concerning the organization and function of the fibrous tissue network of the heart,8 comparatively little is known about the adhesion molecules that maintain valvular ECM homeostasis.

Periostin exhibits structural similarity to fasciclin-I, a Drosophila protein involved in neuronal cell–cell adhesion.9 There are secreted and membrane-associated isoforms of periostin that can act as ligands for select integrins, affecting cell migration, adhesion, and epithelial–mesenchymal transition (EMT) in various normal10,11 and diseased states.12 Periostin can also directly interact with other ECM proteins such as collagen type I, collagen type V, fibronectin, tenasin-C, and heparin,10,13 suggesting periostin plays a...
critical role in ECM homeostasis. Here, we identified perios- tin as a valvulogenesis adhesion molecule marking mesen- chymal cells in both OFT and AV cushions and within mature leaflets following remodeling.\textsuperscript{11,14}

Recently, peristin has been shown to regulate mouse interstitial fibrosis, fibroblast adherence to cardiomyocytes, and ventricular remodeling following pressure overload and myocardial infarction.\textsuperscript{10} Additionally, peristin is upregulated in rat carotid arteries following injury and stimulation with transforming growth factor (TGF)\textsuperscript{\beta}.\textsuperscript{15} and in fibrillin-1 mutant mice exhibiting excessive TGF\textsuperscript{\beta} signaling.\textsuperscript{16} Both systemic TGF\textsuperscript{\beta2} (Tom Doetschman, personnel communication) and cardiomyocyte-restricted \alk3 knockouts exhibit reduced peristin.\textsuperscript{17} Collectively, this suggests that peristin may play multiple roles as a primary responder molecule and be linked to ECM deposition and/or reorganization during homeostasis of adult cardiovascular tissues.

To investigate the role of peristin in hearts, we analyzed targeted null mice\textsuperscript{18} (per\textsuperscript{\textasciitilde}). Our hypothesis was 2-fold: (1) that peristin mediates cushion mesenchyme differentiation into fibroblastic tissue, while inhibiting differentiation into other mesodermal phenotypes; and/or (2) that peristin can act as a collagen-binding protein to modify fibrillogenesis. Here, we show that peristin is dynamically expressed throughout morphogenesis of the fibrous skeleton of the heart and is absent from cardiomyocytes and that expression is upregulated in response to enhanced TGF\textsuperscript{\beta} activity. Both surviving adult and lethal postnatal per\textsuperscript{\textasciitilde}-null valves exhibit a spectrum of ECM structural and molecular abnormalities, notably a blunted fibroblast competence to respond to exogenous TGF\textsuperscript{\beta}, suppressed TGF\textsuperscript{\beta} signaling, and inappropriate differentiation of mesenchymal cushions, resulting in ectopic ECM deposits. Furthermore, peristin is greatly reduced in diseased human valves when the ECM is disorganized. Because a complete loss of heart valve/fibrous skeleton structures is rare in newborn human hearts, these studies highlight the importance of matrix homeostasis in the pathogenesis of valvular/annulus defects.

### Materials and Methods

**Mice**

per\textsuperscript{\textasciitilde} mice\textsuperscript{18} were intercrossed with \alphaMHC-E\textsuperscript{\textasciitilde} reporter mice that express enhanced green fluorescent protein (EGFP) under control of the cardiac-restricted \alpha myosin heavy chain (\alphaMHC) promoter.\textsuperscript{19} Foxc1 mutant embryos were harvested from heterozygous intercrosses,\textsuperscript{20} and Smad6 mutants\textsuperscript{21} were provided by M. Kern (Medical University of South Carolina).

**Gene Expression Assays, Explant Culture, and Histological Analysis**

Histochemistry for \beta-galactosidase, in situ hybridization, RT-PCR, immunohistochemistry, and histology were performed according to standard protocols.\textsuperscript{18} For the cDNA probes, antibodies, and histo- logical stains used, see the online data supplement, available at http://circres.ahajournals.org. Mouse embryonic fibroblast (MEF) isolation, 3D collagen cultures, and TGF\textsuperscript{\beta} responsiveness studies were carried out with as described previously\textsuperscript{22–25} but with minor modifications. See the online supplement for details on procedures.

### Statistical Analysis

Data are presented as means±SD of the mean. Student’s \textit{t} test was applied for data comparison, and \textit{P}<0.05 or \textit{P}<0.01 were assigned as significant.

### Results

**Peristin Exhibits Restricted Expression in Developing Cardiac Skeleton and Mature Valve Apparatus**

In situ analysis revealed that in addition to expression in endocardial cushions and valve leaflets,\textsuperscript{11} peristin is coordi- nately expressed in the developing and mature cardiac fibrous skeleton. Specifically, peristin mRNA and protein are ex- pressed in cardiac fibroblasts, valvular attachment apparatus, chordae tendineae, and epicardial/pericardial structures but are absent from the cardiomyocyte lineage (Figure 1). Significantly, coincident with initial embryonic day (E)10.5 appearance of mouse cardiac fibroblasts,\textsuperscript{8} all 3 embryonically expressed fasciclin-containing genes are expressed (Figure I in the online data supplement). Western blot analysis using a polyclonal \alpha-peristin antibody that detects all known iso-
forms, reveals periostin levels increase coincident with increasing fibroblast content (Figure 1). Protein spatiotemporal expression correlates with mRNA expression in the fibrous skeleton but exhibits a restricted pattern within the leaflets (Figure 1). Restriction occurs before cushion remodeling and fibrous differentiation (supplemental Figure II), suggesting the involvement of periostin in providing a permissive, spatially defined ECM promoting proper organization of nascent cushion ECM. Robust periostin expression is present in mature load-bearing collagenous central fibrosa, suggestive of a structural integrity role during maintenance of unidirectional flow.

**Periostin Is Absent From Cardiomyocytes but Is Expressed Throughout the Rest of the Heart**

Because elevated periostin expression is present following adult myocardial hypertrophy, infarction, arterial injury, and valve calcification, we examined its expression in both normal and heart failure models to assess when and where periostin elevation occurs. To efficiently distinguish cardiac myocyte lineages, we assessed periostin expression and TGFβ responsiveness in cultured E14 cardiac fibroblasts, E14 null fibroblast supernatant was verified via amido-black staining. J, Whereas E14 and newborn fibroblasts express periostin, cardiomyocytes lack expression following RT-PCR analysis. When treated with 1 ng/mL TGFβ overnight, periostin upregulation (7X) is observed in only fibroblasts. Note GAPDH is equally expressed in all samples (n=4 per genotype/age). Scale bars: 10 μm (A and G).

### Figure 2. Periostin expression throughout cardiac fibroblast lineage.

A, B, C, Periostin (A), αMHC-EGFP (B), and 4,6-diamidino-2-phenylindole (DAPI) (C) expression in the same section through adult aMHC-EGFP heart. Periostin is restricted to vascular smooth muscle cells around coronaries (arrow in A) and cardiac fibroblasts (arrowheads in A) but absent from EGFP cardiomyocytes. D, Neonatal heart double-stained with anti-periostin (red) and cardiomyocyte-specific MF20 (green) antibodies (Ab). Fibroblasts (arrows) are periostin-positive, whereas cardiomyocytes are negative. E, Disassociated suspension of 4-day neonatal mouse hearts cocultured for 48 hours on collagen and stained for periostin (red) and sarcomeric actin (green). Note only fibroblasts are red (appear rounded as takes ~5 days to spread on collagen). F, E13.5 right atria and ventricle double-stained with anti-periostin and MF20 antibodies. Note there is no overlap (ie, yellow), and fibrillar-like periostin expression is present only in noncardiomyocyte lineages. G and H, Immunohistochemistry on cultured E14 cardiac fibroblasts reveals both periostin (G) and collagen type I (H) are present in cytoplasm. I, Western blotting showed that E14 cardiac fibroblasts express mainly 82-kDa periostin isoform in cells and secrete larger ~90- and ~87-kDa isoforms. Equal loading of isolated cells and supernatant (supern) was verified via amido-black staining. J, Whereas E14 and newborn fibroblasts express periostin, cardiomyocytes lack expression following RT-PCR analysis. When treated with 1 ng/mL TGFβ overnight, periostin upregulation (7X) is observed in only fibroblasts. Note GAPDH is equally expressed in all samples (n=4 per genotype/age). Scale bars: 10 μm (A and G).

Viable periΔmz Valve Annuli and Leaflets Are Hypoplastic

As expected, histological analysis of E13.5 and E16 null hearts did not reveal structural abnormalities, because periΔmz nulls are present at expected Mendelian ratios at birth. Analysis of surviving adult periΔmz nulls revealing that they often exhibit a bulging right ventricle, particularly within the OFT region, may be indicative of abnormal seminal valves...
are present in null mitral and tricuspid leaflets (supplemental Figure IV). Isolated null leaflets can spontaneously undergo twitching, further suggesting that myocardial tissue is present. This was confirmed via expression of myocardial-specific MF20 in perilacZ-null but not wild-type leaflets (Figure 4E through 4G) but exhibit normal expression of alkaline phosphatase (osteoblast marker), fibulin-1/c/d (aggrecan ligands), and versican (cartilage proteoglycan) expression. Aggrecan is a large chondroitin-sulfate proteoglycan, a major structural component in cartilage matrix also exhibiting dynamic expression in valves.30 Specifically, aggrecan mRNA and protein are localized in the embryo mesenchymal endocardial OFT and AV cushions but downregulated in neonates and absent from normal adult mice hearts.30 Recently, it was proposed that Sox9/Bmp2 regulation of aggrecan may be required for valve remodeling.4 In tendons (closest to valves in terms of tissue type), the relative proportion of collagen:proteoglycan determines tissue type31; thus continued aggrecan expression could result in altered null valve viscoelastic properties or represent lack of appropriate cushion differentiation. Also significant, the related BigH3 gene14 is normally expressed in adult nulls, suggesting it is unlikely BigH3 upregulation compensates for postnatal loss of periostin. However, it is unknown whether BigH3 can functionally compensate for loss of periostin in utero.

To identify where and when perilacZ-null hearts are affected, we assessed in utero morphogenesis and neonatal remodeling. Histology of embryonic (E10 to E12) and fetal (E14 to E18) hearts did not reveal structural anomalies, and Nfatc1 is appropriately expressed in null fetal endocardium overlying the developing endocardial cushions (data not shown). Expression of Nfatc1 is usually extinguished once EMT has occurred,5 indicating that, unlike Foxp1 mutants exhibiting ectopic Nfatc1,32 the perilacZ-null nulls form normally. However, we observed increases in glycosaminoglycans in perilacZ-null E12 cushions (Figure 5B) until E16. Histological analysis of neonatal (1 to 3 weeks) and adult valves revealed loss of trilaminar organization and hypoplastic anuli but unaltered platelet endothelial cell adhesion molecule expression, indicating presence of intact endothelium (Figure 5). Collectively, these results demonstrate that cushion formation is not altered but inappropriate perilacZ-null cushion differentiation and that abnormal late valve remodeling results in viable valvar defects.

Valvular Insufficiency Underlies Neonatal Lethality

In contrast, analysis of nulls dying before weaning (≈14%) revealed gross cardiac architectural and valvar anomalies
nulls and ~50% reduced in heterozygotes. Levels of βigH3, alkaline phosphatase (alp), fibulin-1c/1d are unaltered, but aggrecan is misexpressed in nulls (large*). Aggrecan expression is also dose-dependently upregulated in adult heterozygotes (small*). Significantly, aggrcan (mature and developmentally regulated isoforms) is expressed normally in E13 embryos. F and G, In situ revealed surviving adult perilacZ-null valves (G) continue to express aggrecan, whereas normal littermates (F) have switched it off (n=4).

Periostin Is TGFβ-Responsive

Given that differentiation along inappropriate pathways occurs in the absence of periostin and that it is linked to TGFβ-superfamily signaling, we analyzed Smad6 knockouts. Targeted deletion of the Smad6-negative regulator of TGFβ signaling revealed that it is required for valve maturation and suppression of osteogenesis within endocardial cushions.21 In situ revealed that periostin is upregulated in Smad6 nulls exhibiting valvular hyperplasia (Figure 6A). Thus, altered TGFβ/Smad6 signaling is upstream and may modulate periostin, because when inhibitory Smads are removed (leading to elevated TGFβ signaling), periostin is also upregulated. In support of this, periostin expression is downregulated in Foxc1 nulls failing to undergo TGFβ-mediated mesenchymal maturation (Figure 6A). Foxc1 is a TGFβ1-responsive gene, and null embryos exhibit valve hypoplasia resulting from mesenchymal maturation defects.20 Furthermore, we recently identified a 3.9-kb periostin enhancer containing both TGFβ-responsive and bone morphogenetic protein (BMP)-responsive elements expressed in OFT cushions.23 However, a 304-bp periostin minimal element, still capable of in vivo cushion expression, requires only TGFβ- but not BMP-responsive elements.33 Similarly, we demonstrated Bmp4-null cushions express periostin,25 suggesting that Bmp4 (and possibly other Bmp genes) is not required for periostin OFT expression.

To directly test in vivo TGFβ responsiveness in mature hearts, we assessed expression in cardiac-restricted αMHC-TGFβ1 constitutively active mice.23 Increased TGFβ1-activity was observed during pathological cardiac remodeling in various animal models, but surprisingly, overt fibrosis was only observed in the atria when a TGFβ1 latent complex was tethered to the ECM.34 These data indicate increased TGFβ1 activity alone is insufficient to promote ventricular fibrosis in adult ventricle.34 Supporting our hypothesis, periostin protein
is upregulated in adult TGFβ1 transgenic-positive hearts (Figure 6B), indicating TGFβ1 activation directly correlates with periostin upregulation and can occur without fibrosis. To further assess whether there is altered TGFβ-signaling activity in peri^null^-null leaflets, we performed immunohistochemistry with anti–phosphorylated Smad2,3 antibody, a marker of TGFβ signaling.35 At E14.0, mutant endocardial cushions/leaflets exhibited markedly reduced pSmad2,3 expression before definitive leaflet remodeling (Figure 6E and 6F).

Because we have shown that periostin can directly bind collagen type I,13 we measured collagen synthesis in wild-type and peri^null^-null MEFs alone or coincubated with exogenously supplied TGFβ. When equivalent MEFs were compared, peri^null^-null MEFs alone or coincubated with exogenously supplied TGFβ. When equivalent MEFs were compared, peri^null^-null MEFs synthesized ~32% less collagen than wild types and failed to respond proportionally to added TGFβ (Figure 6C). This indicates that periostin possibly signals fibroblasts to secrete collagen or that it can bind to collagen, thereby decreasing its removal from the ECM. Given the reduced TGFβ-signaling activity and observed ECM disorganization abnormalities in peri^null^-nulls, we evaluated the ability of null MEFs to reorganize and contract 3D collagen lattices and their response to exogenous TGFβ. Collagen gel contraction assays21,24 were used to quantitatively examine the effect of periostin on alignment and condensation of preexisting fibrils analogous to what may be occurring in vivo when cushions become attenuated into cusps by the compactions and organization of fibrils and other ECM components into stratified layers of dense regular fibrous tissue. Significantly, peri^null^-null MEFs exhibit reduced reorganization, contraction ability (Figure 6D), and statistically diminished response to increasing TGFβ (supplemental Fig.-ure VI). This further indicates a defect in the peri^null^-null valvular and nonvalvular fibroblasts that blunts TGFβ responsiveness, resulting in an altered ECM.

Periostin Is Greatly Reduced in Diseased Human Valves

Given the localization of periostin within the collagen-rich fibrosa layer of the valve (Figure 1F), expression studies were extended to human tissue to evaluate periostin in valve disease (Figure 7). In explanted pediatric valve tissue4 from thickened stenotic aortic valves, periostin expression was significantly reduced compared with age-matched controls, correlating with loss of ECM trilaminar stratification, disorganized collagen, and cellular disarray observed in these diseased valves.4 Periostin expression in normal valve tissue extended to human tissue to evaluate periostin in valve disease. This further indicates a defect in the peri^null^-null valvular and nonvalvular fibroblasts that blunts TGFβ responsiveness, resulting in an altered ECM.

Discussion

Although the cellular and molecular mechanisms underlying cushion remodeling and formation of the fibrous skeleton of the heart are complex and depend on multiple gene networks, these data demonstrate periostin is an intriguing matrix effector protein that is required for normal ECM deposition within the heart. Furthermore, not only is periostin directly induced via TGFβ but is required for normal TGFβ signaling and TGFβ responsiveness during in utero cardiac fibroblast,

Figure 6. Periostin is responsive to TGFβ but can also mediate TGFβ responsiveness. A, In situ demonstrated periostin is downregulated in E11.5 Foxc1−/− knockouts and upregulated in E14 Smad6−/− nulls. Note reduced periostin in Foxc1-null cushions, mandibular arch (m), and umbilical (top images) and upregulation in hyperplastic Smad6-null OFT cushions (bottom images). Hematoxylin/eosin staining of transverse sections (middle images) illustrate enlarged OFT cushions and the presence of persistent truncus arterious (*) when inhibitory Smad6 is removed. B, Western blot analysis revealed elevated periostin (9-fold) in 2- and 10-week αMHC-TGFβ1–expressing transgenic ventricles, compared with nontransgenic littersmates. C, 3H-proline incorporation assays indicate collagen production is reduced in null E14 MEFs when compared with wild types (81 vs 8663 vs 5674 ± 94), and they fail to upregulate collagen synthesis with the addition of 1 ng/mL TGFβ (+/− = 11 138 ± 77 vs −/− = 5286 ± 124). Data are expressed as counts per minute of [3H]proline in 10^6 cells (n = 3 independent lines/genotype). D, Three-dimensional collagen lattice contraction after 5 days in response to 0.1 ng/mL TGFβ was compared in 2 independent peri^null^-nulls vs +/+ littermate lines. Note the reduced contraction (~18%) in both nulls, despite the presence of equal cell numbers (10^6 cells/well). E and F, pSmad2,3 is attenuated in E14 peri^null^-null leaflets (F) when compared with +/+ littermate controls (E) (n = 4 per genotype).
valvular ECM organization, and postnatal homeostasis (Figure 8). During myocardial injury, periostin is significantly upregulated coincident with TGFβ-mediated ECM deposition.10,29 Significantly, we demonstrated TGFβ1-induced periostin upregulation can occur in the absence of expression in expanded fibrosa (bar in C) and disorganized ECM, as evidenced by increased valvular interstitial cells and disorganized collagen (arrows in D). Median age of samples is 6 years (range, 10 months to 15 years; n=9) compared with a median age of 9 years for controls (range, 3 to 14 years; n=6). A and C and B and D are viewed at similar magnifications. Scale bars: 1 mm (A); 50 μm (B).

These data do not formally rule out Bmp pathway involvement in regulation of periostin. Because Bmp genes are predominantly expressed in adjacent myocardium and most TGFβ-members are predominantly coexpressed with periostin in cushions themselves, this suggests more likely a direct interaction, compared with the possible indirect effects observed in Alk3 conditional mutants.17 Recent data indicate that periostin may induce reentry of differentiated mammalian cardiomyocytes into the cell cycle.36 However, our mouse10,11,14,17 and chick13 data and nonphysiological truncated form of periostin used28 do not support this. Indeed, expression of full-length periostin in adult rat heart using a plasmid-transfection approach induced disease indices, suggesting the bacterially generated truncated periostin used36 may have had aberrant effects.29

Our analysis revealed that 100% of adult perilacZ-null leaflets are undersized, lack trilaminar ECM organization, misexpress the chondrogenic marker aggrecan, and undergo abnormal differentiation. Notably, lack of periostin resulted in the presence of ectopic smooth muscle and cardiogenic lineages within valves. This is consistent with the islands/pockets of fibrosis reported in adult valves including mice and human37 and Smad6 knockout phenotypes in which cartilage, bone, marrow, and even blood cells were formed within both AV and aortic valves.21 Although it is tempting to speculate that periostin may be required to promote and/or maintain differentiation of condensed mesenchyme into connective tissue and inhibit the chondrogenic and myocardial pathways, it is currently unclear whether genetic redundancy among the fasciclin family obscures the in utero function of periostin or whether loss of periostin expression at the myocardial–endoocardial cushion interface results in irregular delamination, inadvertently trapping nonvalvular cell types. Although the functional consequence of these ectopic cell types has yet to be determined, aspects of these anomalies are similar to those in mice treated with serotonin agonists that increase TGFβ1 activity and collagen biosynthesis, resulting...
in presence of ectopic αSMA cells expressing latent TGFβ-associated peptide. Thus, valves have subtle malformations that may cause latent clinically significant valve disease (eg, fibrillin 1 in Marfan syndrome, TGFBR1/TGFBR2 in Marfan-like disorders, mitral valve prolapse) because small changes over time can cause significant pathology.

Similar to the changes in connective tissue integrity observed in periostin-null nulls represent a useful model of abnormal valve development and often not diagnosed until after birth, the periostin is required for maintenance of periododontal ligament ECM integrity and absorption of mechanical occlusal stress. Because valvular defects are chronic rather than acute and often not diagnosed until after birth, the periostin-null mice exhibit dwarfism, incisor enamel defects, and an early-onset periododontal disease-like phenotype. Mol Cell Biol. 2005;25:11131–11144.


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SUPPLEMENTAL MATERIALS AND METHODS

Supplemental Figure 1. *Fasciclin* family expression in E10.5 hearts. (A) *Periostin*, (B) *βigH3* and (C) *stabilin-1* expression analysis via *in situ* hybridization. Both *periostin* and *βigH3* are co-expressed in cardiac fibroblasts almost as soon as they first appear in mammalian embryonic heart (arrows in A & B). Transmembrane *stabilin-1* gene is expressed in adjacent endothelial lineage (C). Note while *periostin* is confined to endocardial cushion cells of OFT, *βigH3* is expressed in both OFT cushions and adjacent myocardial cuff. Both sense and anti-sense [³⁵S]UTP-radiolabeled RNA probes were transcribed and used for *in situ* hybridization on at least 6-8 mouse embryos at each stage using established techniques¹,². Specific signal was only observed when sections were hybridized with the anti-sense probe.

Supplemental Figure 2. Periostin protein localization in early heart. Immunohistochemistry reveals periostin is expressed in E10 (A,B) endocardial cushions and is co-localized with cardiac jelly matrix and both OFT and AV mesenchymal cushion cells, but is absent from endothelium and adjacent cardiomyocytes. In E11.5 cushions (C,D), periostin protein becomes restricted to cushions cells that abut the adjacent myocardium. Note punctuate expression in cardiac fibroblasts and within epicardium (arrow in C). Periostin is robustly expressed in both the E13 aortic valve leaflets and valvular attachment apparatus (arrow in E), as well as the mitral valve leaflets (F). Immunostaining of periostin (1:10,000
Supplemental Figure 2: Elevated periostin in failing hearts is confined to cardiac fibroblast lineage. To assess the pathological responses of periostin, we examined periostin responses to pressure overload, fibrosis and myocarditis. Acute LV pressure overload was created by microsurgical transverse aortic constriction. At 2 and 7 days post-surgery, constricted and sham-operated heart were removed and LV weight determined. The LV weight...
was indexed to body weight to verify expected heart weight increase in only the 7-day constricted animals\textsuperscript{4}. Adult hearts were then perfusion-fixed with phosphate buffered saline (PBS)/4\% PFA and dissected to enable morphometric and marker analysis as wholemounts and histological sections. Heart samples were harvested, fixed, sectioned (6µm), stained with hematoxylin and eosin (H&E for cardiac histology), Sirius red/Fast green (for potential cardiac fibrosis), von Kossa (detect calcification), and Resorcin-Fuchsin/van Gieson (stain collagen & elastin fibers).

Significantly, in ventricular samples that had been subjected to acute LV pressure overload, the \textasciitilde 85, 87 and 90kDa periostin isoforms are upregulated specifically within cardiac fibroblasts 7 days after banding (64x fold). Protein levels were normalized by amido black staining (n=4 banded LV and 4 sham-operated LV pooled samples; scale bars: 50µm). Periostin is upregulated (64x) in banded samples 7 days post-surgery and coincident with development of fibrous (SFig. 3A). However, periostin is unchanged in banded hearts 2 days post-surgery (not shown). Furthermore, spatiotemporal upregulation is only observed in the cardiac fibroblast lineage and in regions of overt fibrosus (SFig. 3D & E). These findings are consistent with microarray results that profiled genetic responses during infarction\textsuperscript{5}, and indicate upregulation is within the cardiac fibroblast lineage occurring within 1-week of infarction. (B & D) Cryostat sections through ventricles of 7day banded adult heart. B & D panels show sections, stained with Sirius red (stains fibrotic regions) and fast green (stains cardiomyocytes). Note fibrosis and abundant collagen deposition (red signal) throughout the banded heart. (C & E) Adjacent sections stained for periostin protein expression (brown DAB staining). Note up-regulation of expression in only cardiac fibroblasts in banded failing hearts.

Given the robust periostin upregulation observed in response to aortic banding, we assessed the spatiotemporal expression of periostin in a myocarditis model – namely DBA/2 inbred mice (SFig. 3F-H). DBA/2 mice spontaneously develop myocarditis and a unique form of subepicardial inflammation of the right ventricle characterized by a prominent eosinophilic infiltrate with calcinosis\textsuperscript{6}. The myocardial injury is most severe \textasciitilde 7 weeks but heals with myocardial fibrosis and calcinosis \textasciitilde 10 days later. Western analysis revealed periostin levels were unchanged in 4-week DBA/2 hearts prior to myocarditis but significantly increased (23x fold) in 8-week fibrotic hearts (not shown). Immunohistochemistry revealed periostin upregulation is most evident in the fibrotic regions, surrounding calcified nodules but absent
from the cardiomyocytes. Analysis of 7-week DBA/2 hearts exhibiting myocardial fibrosis, necrosis and calcinosi revealed that upregulated periostin expression is confined to the regions of calcification, restricted to the activated cardiac fibroblasts and is also absent from cardiomyocytes (SFig. 3F-H). (F) Von Kossa staining shows subepicardial mineralization (black), while (G) Sirius red indicates fibrosis at site of myocardial injury. Note periostin (brown DAB staining) immuno-detection (H) demonstrates that periostin expression overlaps region of calcification and is restricted to the activated cardiac fibroblasts. (n=5 hearts examined). Our data, and that shown by Molkentin et al.\textsuperscript{5} and Markwald et al.\textsuperscript{7} clearly demonstrate that periostin is not expressed in cardiomyocytes in either the normal, failing and/or dilated mice hearts. Thus, despite both acute LV pressure overload and myocarditis causing significant ventricular fibrosis, abundant collagen deposition and significant periostin upregulation; periostin expression is confined to the cardiac skeleton and valves,
Viable $\text{per}^{\text{lacZ}}$ valve annuli and leaflets are hypoplastic: Histology reveals 100% of null (n=8/8 sectioned) mitral and tricuspid leaflets are significantly shorter (relative lengths indicated by green dotted line in H&E sections; wildtype=2,232±18±18µm vs. null=1,306±51µm; P<0.05) and thickened compared to wildtype littermates (Fig. 3b). Subsequent analysis of proliferation index and cell density in wildtype and $\text{per}^{\text{lacZ}}$ null littermates containing the $\alpha\text{MHC-EGFP}$ reporter, indicate null cushions are initially formed normally, and exhibit equivalent proliferation and have similar cell densities, compared to wildtypes. When DAPI-positive EGFP-negative nuclei, that stained positively for BrdU, were compared in E14 cushions (n=4; +/+ was 12.4±0.7%; $\text{per}^{\text{lacZ}}$ null was 11.2±4%), newborn (n=3; +/+ was 1.7±0.8%; $\text{per}^{\text{lacZ}}$ null was 1.9±3%) and four month leaflets (n=3; +/+ was 0.06+/-.01%; $\text{per}^{\text{lacZ}}$ null was 0.043+/-.007%), there were no statistically significant differences. As normal adult cardiomyocytes have a labeling index of ~0.0005%, the more than 100-fold higher proliferative index suggests postnatal valves remain active, dynamic structures. Furthermore, histomorphometric analysis of four month old $\text{per}^{\text{lacZ}}$ null and littermate control hearts (n=6 of each genotype), revealed that DAPI-positive, but EGFP-negative fibroblast cell numbers were unaffected by loss of periostin (+/+ fibroblast content was 81.4+/2.8% of total cells, n=2,657 cells; and $\text{per}^{\text{lacZ}}$ null fibroblast content was 74.7+/1.65% of total cells, n=3,431 cells counted). Similarly, total cellularity of four month old wildtype ventricle was 1,224 cells/1,4mm² (n=6) and $\text{per}^{\text{lacZ}}$ null was 1,127 cells/1,4mm² (n=6), indicating no significant differences in global cell number, despite systemic periostin loss.

Supplemental Figure 4. Mesenchymal cushion differentiation is perturbed in absence of Periostin. To visualize the cardiac skeleton, we made use of the $\alpha\text{MHC-EGFP}$ reporter mice to distinguish EGFP-positive cardiac myocytes from other cell types in the mature heart. Significantly, the adult surviving $\text{per}^{\text{lacZ}}$ null short leaflets contained ectopic islands of $\alpha\text{MHC-EGFP}$ cardiomyocytes, as isolated EGFP-positive cells are present in null mitral and tricuspid leaflets (SFig. 4). Similarly, ectopic $\alpha\text{SMA}$-expression is also present in adult null valves. (A-C) Phase (A) and EGFP (B&C and inset) images of 4-month adult wildtype (A&B) and $\text{per}^{\text{lacZ}}$ null (C & enlarged inset) hearts containing the $\alpha\text{MHC-EGFP}$ transgenic reporter. Note ectopic EGFP-positive myocyte islands are present in null valves (n=9/13 $\text{per}^{\text{lacZ}}$ null $\alpha\text{MHC-EGFP}$ hearts examined). (D,E) $\alpha\text{SMA}$ expression in wildtype (D) and $\text{per}^{\text{lacZ}}$ null (E & enlarged
inset). Note ectopic actin-expressing clusters in short null leaflets (arrow in inset; n=3/13 peri$^{lacZ}$ null αMHC-EGFP hearts examined).

**Supplemental Figure 5. 3D-collagen lattice formation results.** Collagen gel contraction assays were employed specifically to quantitatively examine the effect of periostin on alignment and condensation of pre-existing fibrils analogous to what might be occurring in vivo when cushions become attenuated into cusps by the compactions and organization of fibrils and other ECM components into stratified layers of dense regular fibrous tissue – rather than study fibrillogenesis itself. This assay enables us to assess the mechanism and function and significance of periostin binding to collagen. To evaluate the ability of fibroblasts to reorganize and contract 3D-collagen lattices and their response to exogenously added TGFβ, wildtype and peri$^{lacZ}$ null E14 MEFs were cast into floating collagen lattices, and daily contraction measured, as previously described$^{9,10}$. Similarly, TGFβ responses of wildtype and peri$^{lacZ}$ null E14 MEF lines were also compared.

Whilst wildtype collagen lattices rapidly contracted from a starting diameter of 3.5cm to less than 2cm over 5 days, the peri$^{lacZ}$ null fibroblasts exhibit reduced reorganization and contraction ability (SFig. 5). The average degree of contraction in 3 independent cell lines per genotype (measured as the gel area as a percentage of the original area) for wildtype MEFs was 42% (day1), 78% (day2), 84% (day3) 91% (day4) 100% (day 5); while the mutant was
12% (day 1), 20% (day 2), 33% (day 3) 52% (day 4) 52% (day 5). Similarly, when wildtype lattices were exposed to increasing TGFβ (0.1 and 1 ng/ml) they contracted significantly more than untreated lattices cultured for the same length of time. When lattices seeded with peri lacZ null MEFs were compared with wildtype in the presence of exogenously added 0.1 or 1 ng/ml TGFβ (SFIG. 5), the peri lacZ null fibroblasts exhibit reduced reorganization and contraction ability (null gels were ~51% larger than wildtype gels; for all gel contraction experiments P<0.05 was considered statistically significant). This supports the hypothesis that loss of perioptin results in a blunted TGFβ ECM response.

METHODS:

Animal models: Periostin (peri lacZ) knockin knockout mice generated previously were maintained on a C57BL/6J genetic background and fed powdered Teklad LM-485 Complete Mouse Diet to alleviate runting11. Peri lacZ mice were intercrossed with αMHC-EGFP reporter mice12 that express EGFP under the control of the cardiomyocyte-restricted α myosin heavy chain (αMHC) promoter. To assess TGFβ responsiveness, periostin expression was measured in αMHC-TGFβ1 mice13, that express constitutively active mutant TGFβ1 cys33ser. To assess
the pathological responses of periostin, acute left ventricular (LV) pressure overload and sham-operated control samples were created by microsurgical transverse aortic constriction as described previously\(^4\). Adult DBA/2 inbred mice that spontaneously develop myocarditis\(^6\) were purchased from Jackson Laboratories and periostin examined. All animal experimentation was performed in accordance with National Institutes of Health Guidelines, and protocols approved by the Institutional Animal Care and Use Committee at IUPUI.

**Histological, in situ, RT-PCR and immunohistochemical analysis:** Tissue isolation, fixation and processing for lacZ staining was carried out as described\(^{11}\). Histomorphometric and proliferation analysis of peri\(^{lacZ}\) hearts are standard and detailed in supplement. Sections (12µm) and microdissected tissues were subjected to S\(^{35}\) in situ hybridization and immunohistochemical analyses of matrix-bound and secreted periostin was carried out as described\(^{1,2,5,11}\). Pediatric aortic valve tissue was obtained as described\(^{13}\). Resorcin-Fuchsins/vanGieson staining was used to detect collagen/elastin and alcian blue (pH2.5) to detect glycosaminoglycans; and αSMA (Sigma), collagen-I (SouthernBiotech), Ddr2 (Santa Cruz), MF20 (Hybridoma bank), and αsarcomeric actin (Sigma) antibodies were used to assess lineage-restricted periostin expression. Phospho-Smad2,3 antibody (Cell Signaling) was used to assess Tgfβ signaling.

**Histomorphometric and proliferation analysis:** Four month old peri\(^{lacZ}\) null and age matched littermate control hearts containing the αMHC-EGFP reporter were serially sectioned (6µm) and stained with 4’,6-diamidino-2 phenylindole (DAPI) to demonstrate nuclei via fluorescence microscopy (n=6 hearts/genotype). Histomorphometric analysis was performed on digital images (via ImageJ software, NIH) to assess relative cardiac fibroblast cell numbers and total ventricular cellularity. EGFP-positive cells containing DAPI nuclei and total DAPI stained nuclei were counted from 4 random fields (at 200x mag) per slide and 2 slides/ventricle. Leaflet lengths were measured between the anchoring point to the annulus fibrosus and the free edge as described\(^{14}\). Valvular attachment site areas were determined via measurement of the DAPI stained and αMHC-EGFP negative area using combined phase/fluorescence microscopy. Incorporated (BrdU at 1ml/100g, ZYMED) immunopositive cells/compared to DAPI-stained
total, were counted from 4 random fields (200x mag) per slide and 2 slides/heart, with total of 6 hearts per group for statistically testable analyses.

**Isolation and Culture of Cells:** For TGFβ-responsiveness studies, wildtype and peri<sup>lacZ</sup> null cardiomyocytes and fibroblasts were obtained from collagenase digestion of E14 and newborn hearts, as described for neonatal hearts<sup>15</sup> except digestion times were reduced for fetal hearts. Fibroblasts were separated from myocytes by selective cellular attachment, and myocytes were further purified by using a Percoll density gradient (Sigma). Fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% NBS, 5% FCS, 1% penicillin/streptomycin (Sigma). Myocytes were maintained in DMEM containing 8% horse serum (Gibco), 5% NBS, 1% penicillin/streptomycin. Cardiac fibroblasts and myocytes were cultured alone or co-incubated with TGFβ (1ng/ml; Gibco) for 16hrs, and used for RT-PCR as described<sup>1,2,11</sup>.

MEFs were isolated from wildtype and peri<sup>lacZ</sup> null E14 embryos, and used to assess collagen production and 3D-lattice formation ability, using the methods described<sup>9,10</sup>. Collagen production by wildtype and peri<sup>lacZ</sup> null MEFs alone or co-incubated with TGFβ (1ng/ml; Gibco) in 24-well tissue culture plates was assessed by [<sup>3</sup>H]proline incorporation. Equivalent MEFs (10<sup>5</sup> cells from 3 independent cell lines per genotype) were assessed as described<sup>10</sup>.

For assessment of 3D-lattice formation ability, collagen type-I from rat tail tendon (2mg/ml in 18mM acetic acid) was added to 35mm dishes containing DMEM, 1% pen/strep with 1% FCS and 0.1M NaOH. Wildtype and peri<sup>lacZ</sup> null MEFs (10<sup>5</sup> cells/well) were added just after collagen solution, before fibrillation and lattice formation. The relaxed, free-floating gels were incubated at 37°C and the media (+/- 0.1 and 1ng/ml TGFβ) changed daily. The lattices were photographed and rates of gel contraction were calculated by determining the remaining surface area by computer-based analysis and expressed as either percentage of initial area or percentage of control area. Data are presented graphically as mean +/-sd. Lattice area was statistically compared with Student’s t test. Significant differences were determined at P<0.05.
Literature cited:


