Epicardial-Derived Cell Epithelial to Mesenchymal Transition and Fate Specification Require PDGF Receptor Signaling

Christopher L. Smith, Seung Tae Baek, Caroline Y. Sung, Michelle D. Tallquist

Rationale: In early heart development, platelet-derived growth factor (PDGF) receptor expression in the heart ventricles is restricted to the epicardium. Previously, we showed that PDGFRβ is required for coronary vascular smooth muscle cell (cVSMC) development, but a role for PDGFRα has not been identified. Therefore, we investigated the combined and independent roles of these receptors in epicardial development.

Objective: To understand the contribution of PDGF receptors in epicardial development and epicardial-derived cell fate determination.

Methods and Results: By generating mice with epicardial-specific deletion of the PDGF receptors, we found that epicardial epithelial-to-mesenchymal transition (EMT) was defective. Sox9, an SRY-related transcription factor, was reduced in PDGF receptor-deficient epicardial cells, and overexpression of Sox9 restored epicardial migration, actin reorganization, and EMT gene expression profiles. The failure of epicardial EMT resulted in hearts that lacked epicardial-derived cardiac fibroblasts and cVSMC. Loss of PDGFRα resulted in a specific disruption of cardiac fibroblast development, whereas cVSMC development was unperturbed.

Conclusions: Signaling through both PDGF receptors is necessary for epicardial EMT and formation of epicardial–mesenchymal derivatives. PDGF receptors also have independent functions in the development of specific epicardial-derived cell fates. (Circ Res. 2011;108:00-00.)

Key Words: epicardium ■ PDGF ■ cardiac fibroblast ■ EMT ■ sox9

Cardiac disease is the leading cause of death in the industrial world. Although recent stem cell therapies have attempted to regenerate myocardium, there are still many physiological barriers to overcome, including fibrosis, inflammation, and insufficient blood vessel generation. Induction of cardiomyocyte regeneration is one proposed way to improve cardiac function, but it is clear that the noncardiomyocyte populations in the heart also contribute to the repair process. Noncardiomyocyte lineages (endothelial cells, vascular smooth muscle cells, and cardiac fibroblasts) are essential for blood vessel formation and matrix organization, and an understanding of the developmental signals that shape these cells may provide insights into disease pathogenesis and better heart injury therapies.

Coronary vascular smooth muscle cells (cVSMC) and cardiac fibroblasts develop from the epicardium in a multistep process involving cell proliferation, epithelial-to-mesenchymal transition (EMT), and mesenchymal cell fate specification. Several proteins have been implicated in the development of cVSMC from the epicardium, but less is known about the epicardial-derived cardiac fibroblast population. It is proposed that cardiac fibroblasts are essential for normal cardiac function, and their role in matrix deposition during cardiac injury is well established. Yet, signaling pathways regulating their development are poorly understood.

Platelet-derived growth factor (PDGF) receptor tyrosine kinases are important for embryonic development and play essential roles in the forming vasculature. Previously, we identified a role for PDGFRβ as an important factor regulating epicardial-derived cVSMC development. We and others observed PDGFRα in the epicardium; however, no data exist regarding the fate of epicardial-derived cells (EPDCs) when PDGFRα is disrupted. The receptors are coexpressed in the epicardium until E13.5, but after this time point, receptor expression becomes mutually exclusive. These initial findings led us to investigate the role of PDGFRα individually and combined with PDGFRβ during EPDC formation.

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Nonstandard Abbreviations and Acronyms

cVSMC: coronary vascular smooth muscle cell
EKO: epicardial knockout
EMT: epithelial-to-mesenchymal transition
EPDC: epicardial-derived cell
epi: epicardium
LV: left ventricle
myo: myocardium
PDGF: platelet-derived growth factor
pm: papillary muscle
RV: right ventricle
SEM: subepicardial mesenchyme

Using cre/loxP recombination, we generated animals that lacked PDGFRα, PDGFRβ, or both PDGF receptors in the epicardium. Epicardial deletion of both PDGF receptors resulted in failure of epicardial EMT and EPDC formation. Loss of PDGF signaling led to reduced Sox9 expression, and when Sox9 expression was restored in mutant hearts, the EMT defect was rescued. Interestingly, mutants lacking only one of the PDGF receptor genes exhibited a lineage specific requirement for each individual receptor. Loss of PDGFRα resulted in a deficit in cardiac fibroblast formation, whereas cVSMC development was unperturbed. Conversely, loss of PDGFRβ resulted in failure of epicardial EMT and EPDC formation. Combined, our data demonstrate a novel role for PDGF receptors in epicardial EMT and EPDC development.

Methods

Additional methods are available in the Online Data Supplement at http://circres.ahajournals.org.

Results

Loss of Both PDGF Receptors Causes Defects in Epicardial Cell Migration

Both PDGF receptors, α and β, are expressed by the epicardium, and loss of PDGFRβ alone causes a reduction in cVSMC.4 To investigate a combined role for PDGF receptor signaling, we deleted PDGF receptors from epicardial cells using mice expressing the Gata5Cre transgene.16 PDGFREKO (EKO, or epicardial knockout) hearts had regions of epicardial detachment and hemorrhaging (Figure 1A). The detachment progressed temporally from the dorsal to ventral heart surface but resolved by birth (data not shown). Despite this phenotype, the epicardium expressed multiple, established epicardial markers (Online Figure IA and IB). Because PDGF receptor signaling has been associated with proliferation and survival,17 we examined the epicardium and EPDCs for BrdU incorporation and cleaved caspase-3 activation. PDGFREKO mutant values were similar to those obtained in controls (Online Figure IC and ID), suggesting that loss of PDGF receptor

Mice were maintained on a mixed C57Bl6×129Sv background. The strains in these experiments included PDGFREFP, PDGFREβ, PDGFREβ, R26RFP, R26Rt2 (Jackson Labs), R26RlacZ, WT1CRE TIE2CreTop, and Gata5Cre Tg6.16 All animal protocols and experiments were approved by the UTSW IACUC and conformed to National Institutes of Health guidelines for care and use of laboratory animals. Gata5Cre transgenic and WT1CRE mice were kindly provided by Dr Ruiz Pilar-Lozano (Burnham Institute) and Dr William Pu (Harvard University), respectively. WT1Cre−/− animals were induced with tamoxifen at indicated time points. Tamoxifen (MP Biomedicals, 0215673894) was dissolved in sunflower seed oil (Sima, S5007) at 20 mg/mL. We administered 0.1 mg/g body weight of tamoxifen by oral gavage. Controls used in most experiments were Gata5Cre-negative littermates. Animals used for controls in fibroblast isolation(s) were PDGFREFP, PDGFREβ, R26RlacZ, and Gata5Cre+.

Figure 1. PDGFREKO epicardial cells fail to migrate into the myocardium. A, Whole-mount images showing regions of epicardial detachment and hemorrhaging (arrows). B, R26RFP IHC was used to examine epicardial cell migration into subepicardial mesenchyme (brackets) from indicated genotypes induced with tamoxifen at E12.5. Arrowheads point to migrated cells within the subepicardial mesenchyme. C, Quantification of the R26RFP fluorescent area in B. N values are indicated in parentheses. *P<0.005. D, Quantification of GFP+ cells within myocardium of E12.5 hearts transduced with an adenovirus expressing GFP and stimulated with hTGFβ or bFGF (n=3 for each genotype/condition). Data are represented as mean±SD. *P<0.001 (in comparison with vehicle-treated control). LV, left ventricle, RV, right ventricle, EKO, epicardial knockout.
signaling does not affect cellular proliferation or survival of epicardial cells in vivo.

Because epicardial formation was unaffected in PDGFREKO mutants, we next assayed hearts for epicardial cell entry into the myocardium. To trace epicardium lacking PDGF receptors, we induced cre-recombinated recombination in epicardial cells just prior to EMT (E12.5) using a tamoxifen-inducible WTI^{RFP} allele and analyzed migration at E14.5. Using R26R^{YFP} reporter activity to follow the epicardial cells, we observed a loss of EPDCs when PDGF receptors were absent (Figure 1B and 1C). Similar results were obtained when using markers of undifferentiated EPDCs, WT1^{18,19} or mesenchymal cells, vimentin. Both markers showed a severe reduction in the region immediately underlying the epicardium in PDGFREKO hearts (Online Figure II and data not shown). Note that vimentin is a broad mesenchymal marker that is also expressed by nonepicardial-derived coronary endothelial cells. Additionally, in an ex vivo migration assay, fewer cells exited the epicardium in PDGFREKO mutant hearts even when stimulated with EMT-inducing growth factors, hTGFβ1, and bFGF (Figure 1D).

**PDGF Receptor Signaling Is Required for Epicardial Cell EMT**

We surmised that loss of epicardial cell migration was caused by a defect in epicardial EMT. We first examined PDGFREKO embryonic hearts for expression of transcriptional inducers of EMT, Snail, Slug, and Sox9. Interestingly, even though epicardial genes—such as WTI and Tbx18—were unchanged, we consistently observed a significant reduction in Snail, Slug, and Sox9 transcript levels (Figure 2A).

EMT involves a complex series of events including the loss of epithelial morphology and the acquisition of mesenchymal actin filaments. Therefore, we examined EMT induction in primary epicardial cultures using a cocktail of growth factors. We compared cell morphology by bright field imaging and immunostaining for adherens junctions and filamentous actin organization (β-catenin and phalloidin, respectively). While control cultures lost their epithelial characteristics (junctional β-catenin) and gained mesenchymal cell morphology (cytoplasmic actin stress fibers), PDGFREKO mutant cultures remained epithelial, illustrating a failure to initiate EMT (Figure 2B, Online Figure IIIA).

EMT is also associated with changes in gene expression. To evaluate additional EMT markers, we performed qPCR analysis. Initial experiments revealed that primary cultures undergo EMT, but many of the genes commonly used to assess EMT were not significantly altered in the stimulated epicardial cultures. For example, we observed no changes in E-cadherin, ZO-1, αSMA, and vimentin expression (data not shown). Therefore, to generate an EMT profile specific for primary epicardial cultures, we performed gene expression analysis on E12.5 cultures treated with vehicle or hTGFβ1 (10 ng/mL), PDGFBB (20 ng/mL), and bFGF (25 ng/mL), all 3 being growth factors known to stimulate EMT (GEO Series GSE27181). From these data, we generated a list of candidate genes and verified a subset that correlated with a change from an epithelial to a mesenchymal phenotype. Two epithelial markers that were consistently downregulated on EMT induction were Krt14 and BVES. We also identified a group of mesenchymal genes that were induced on EMT induction. This list included Calponin, Snail, Sox9, Cdh6, Cal7α, MMP10, and OPG.

Having established these gene sets, we then investigated their expression during the EMT response in mutant cultures. For every gene examined, we found that expression in the mutant cultures was significantly different from stimulated control cultures (Figure 2C through 2E). Interestingly, vehicle-treated mutant cultures consistently exhibited increased levels of the epithelial gene, Krt14. These data suggest that a defect in the process of EMT was present in PDGFREKO epicardial cells.

**Expression of Sox9 in PDGFREKO Cells Rescues the EMT Defect**

To determine potential genes that mediate PDGF driven EMT, we screened for gene expression differences using microarray data sets from whole hearts and primary epicardial cultures (GEO Series GSE27181). Comparison of control and PDGFREKO data demonstrated that transcripts of an SRY-related family member, Sox9, were decreased in mutant E12.5 and E13.5 hearts and in primary epicardial cultures (Figure 2A, data not shown).

The correlation of Sox9 transcript levels with PDGF signaling led us to investigate a role for Sox9 in PDGF-dependent EMT. In primary epicardial cultures, PDGF stimulation resulted in increased Sox9 expression (Figure 3A). We next determined how Sox9-induced expression impacted these cultures. In the absence of stimulation, Sox9 overexpression had little effect on the cultures, regardless of the genotype. However, AdSox9-transduced cultures stimulated with hTGFβ1 and PDGFBB changed from an epithelial morphology to a mesenchymal morphology (Figure 3B, Online Figure IIIB). These data suggested that additional signaling pathways were required to initiate a Sox9-mediated EMT in our primary epicardial cell cultures, similar to what has been observed in neural crest cells but that Sox9 expression could induce EMT even in PDGFREKO epicardial cells.

To examine Sox9’s role in epicardial EMT gene expression, we transduced cultures with AdSox9 (Figure 3C through 3E). These cultures had reduced epithelial (BVES and Krt14) gene expression, but mesenchymal gene expression remained unchanged. However, similar to the morphological assay, AdSox9 transduced and stimulated (hTGFβ1 and PDGFBB) cultures had both decreased epithelial gene expression and increased mesenchymal gene expression regardless of genotype (Figure 3C through 3E). Adenoviral transduction alone did not change the gene expression profile of epicardial cells (Online Figure IIID through IIIF).
Figure 2. PDGFR<sup>EKO</sup> epicardial cells fail to undergo epithelial-to-mesenchymal transition (EMT). A, The qPCR analysis of gene expression in PDGFR<sup>EKO</sup> whole hearts (atria and conotruncal regions removed) for transcriptional EMT markers (Snail, Slug, Sox9) and epicardial markers (Tbx18, WT1, PDGFR<sub>α</sub>, PDGFR<sub>β</sub>). Data were compared with control littermates and are representative of 3 independent experiments. *P<0.001. B, The β-catenin and phalloidin localization in primary epicardial cultures after 48 hours of stimulation with hTGFβ1 and PDGFBB. C through E, The qPCR on primary cultures for expression of epithelial, transcriptional, and mesenchymal markers. Data were compared with vehicle-treated control epicardial cultures (represented by a baseline of 1.0). Data are represented as mean±SD. N values are indicated in parentheses.
Because Sox9 expression has not been documented in the epicardium previously, we examined hearts for Sox9 protein. Sox9 was present in a subpopulation of epicardial cells at E13.5 (Figure 4A), and a day later, Sox9 cells were present in both the epicardium and subepicardial mesenchyme (Figure 4A). Using the WT1\textsuperscript{Cre} and R26\textsuperscript{RFP} alleles, we confirmed that Sox9 cells are epicardial derived (Online Figure IVA). In contrast to controls, Sox9 expression in PDGFRE\textsuperscript{EXO} hearts was significantly reduced at E13.5 and virtually absent at E14.5 (Figure 4A and 4B). We observed a PDGF receptor gene dosage effect on Sox9-expressing cells that correlated with the number of functional PDGF receptor alleles present (Online Figure IVB). Individual PDGF receptor epicardial mutants also contained reduced numbers of Sox9\textsuperscript{+} cells (Figure 4B), suggesting that signaling from either receptor is involved in Sox9 expression.

To determine whether Sox9 could rescue the PDGFRE\textsuperscript{EXO} epicardial migration defect, we transduced control and PDGFRE\textsuperscript{EXO} hearts with AdGFP and AdSox9 (Figure 4C). AdSox9 transduction was able to induce migration of epicardial cells from both control and PDGFRE\textsuperscript{EXO} hearts. This result suggested that, in whole-heart cultures, Sox9 was sufficient for inducing epicardial EMT but not in
isolated epicardial cultures. The myocardium may provide additional cues in vivo. Taken together, these results implicate a role for Sox9 in PDGF receptor-dependent EMT and demonstrate that Sox9 can partially rescue the EMT defect caused by PDGF receptor deletion in epicardial cells.

Loss of PDGFRα Leads to an EMT Defect in a Subpopulation of Epicardial Cells

Our data suggested that both PDGF receptors are required for EMT, but results from the PDGFRβ epicardial deletion demonstrated a VSMC lineage defect in epicardial development.4 Therefore, we decided to further investigate the individual role of the PDGF receptors during EPDC development. To obtain a more precise view on PDGF receptor expression overlap, we used flow cytometry. We observed that both receptors were initially coexpressed at early embryonic stages (E13.5) but became mutually exclusive at later stages (E16.5) (Online Figure V). With a GFP knock-in allele7 to follow cells expressing PDGFRα, GFP expression appeared in most epicardial cells at E13.5 (Figure 5A). At E13.5, PDGFRα/GFP/GFP mutant hearts exhibited epicardial blistering that was milder than PDGFRβ/GFP/EKO hearts (data not shown). However, this blistering was less severe than that observed in embryos null for a different PDGFRα allele.40 The phenotypic differences described for the PDGFRα null embryos could be caused by differences in genetic background or by the fact that 2 of these studies (this report and one by Bax et al41) excluded embryos that had retarded growth from analysis. With regard to the number of GFP+ cells in the epicardium, we observed no differences between control and mutant hearts (Figure 5A). However, at E17.5, GFP+ cells were present within the myocardium of heterozygote hearts, but no GFP-expressing cells were observed within the myocardium of PDGFRα/GFP/GFP hearts. These data suggested that loss of PDGFRα signaling leads to a disruption of a cell population that might arise from the epicardium and is consistent with a recent report showing reduced WT1+ cell migration in PDGFRα/GFP/GFP hearts.41

To determine whether the loss of PDGFRα-expressing cells in the myocardium of the null was caused by a failure in PDGFRα-dependent cell migration from the epicardium, we deleted PDGFRα in epicardial cells. PDGFRα/GFP/EKO hearts showed a reduction of GFP expressing cells within the myocardium, similar to PDGFRα/GFP/GFP animals (Figure 5B). Lineage tracing at E12.5 using an inducible, epicardial-specific Cre mouse line (WT1iCre)14 and R26RtdT12 demonstrated that GFP-expressing cells were epicardial derived. In addition, when PDGFRα epicardial function was disrupted, migration of PDGFRα/GFP positive cells into the heart was reduced (Figure 5C and 5D).

**PDGFRα Mutant Hearts Have a Selective Loss of Cardiac Fibroblasts**

We next examined whether there was a defect in the formation of epicardial derivatives in the absence of PDGF...
receptor signaling. Because epicardial EMT was disrupted, we expected aberrant cVSMC and cardiac fibroblast development. Surprisingly, the expected Mendelian ratio of PDGFRα/H9251 EKO, PDGFRα/H9252 EKO, and PDGFREKO mutant animals was recovered at weaning and up to 1 year after birth. No measurable defects in cardiac size or function were observed (Online Figure VI). Loss of PDGFRα/H9252 alone results in an absence of epicardial-derived cVSMC, but a secondary population of cVSMC is initially present at the aortic root,4 which continues to expand as the animals age (data not shown). This rescue may explain why loss of epicardial cVSMC does not lead to lethality. Examination of the endothelial component of the coronary vasculature suggested that patterning of the vessels in the PDGFREKO hearts was similar to that previously reported for the PDGFRα/H9252 EKO,4 and that endothelial cell presence within the heart was not disrupted by a lack of EPDCs. We determined the consequences of disrupted epicardial EMT by examining hearts for epicardial derivatives. Staining for cVSMC markers, SM22α, smooth muscle myosin heavy chain (SMMHC), α-smooth muscle actin, and PDGFRβ demonstrated that cVSMC content of PDGFRα/EKO hearts was unaffected (Figure 5E and data not shown). Because the smooth muscle cell markers that we examined should detect VSMC as well as pericytes, we conclude that loss of PDGFRα does not affect the mural cell lineage. By contrast, PDGFREKO hearts showed a reduction in all of these markers (data not shown), similar to the loss that we reported in PDGFRα mutant hearts. These data, in combination with the observed loss of GFP+ cells, suggested that PDGFRα might be required for the formation of a distinct EPDC population, cardiac fibroblasts.

To determine whether PDGFRα was required for cardiac fibroblast formation, we analyzed R26R<sup>YFP</sup> epicardial lineage tagged hearts for a cardiac fibroblast surface marker, Thy1.42–44. Epicardial-derived fibroblasts were defined as YFP+, Thy1+, and CD31+ (Figure 6A). Deletion of PDGFRα either individually or in combination with PDGFRβ (PDGFRα<sup>EKO</sup> and PDGFREKO, respectively) resulted in a loss of epicardial-derived cardiac fibroblasts and an absence of YFP+ cells in PDGFREKO hearts. Epicardial-derived fibroblast numbers in hearts lacking PDGFRβ (PDGFREKO) were similar to controls (Figure 6A). Next, we generated primary cardiac fibroblast cultures and traced the epicardial lineage using Gata5Cre transgene and an R26R<sup>LacZ</sup> allele to identify EPDCs.
Primary cardiac fibroblasts isolated from PDGFREKO and PDGFRAEKO hearts had a paucity of epicardial-derived cardiac fibroblasts (β-galactosidase−). By contrast, the number of epicardial-derived cardiac fibroblasts observed in controls and PDGFRIKO hearts were very similar (Figure 6B and 6C).

Because a population of cells grew from PDGFREKO and PDGFRAEKO primary fibroblast cultures, we surveyed hearts for overall fibroblast content by detecting transcripts of fibroblast enriched genes. The qPCR demonstrated, on average, a 50% reduction in fibroblast gene transcripts in PDGFREKO hearts (Figure 7E). We then used Collal (Figure 7A and 7B) and prolyl-4-hydroxylase β (P4hb) expression perinatally. Cells expressing these 2 genes were evident from E18.5 to P7, but after P7, in situ detection of gene expression appeared to taper off (Online Figure VIIA). These data suggested that matrix production by epicardial fibroblasts fell within a very discrete time window. A greater than 50% reduction was observed in the fibroblast population of PDGFREKO at P7, and a reduction in these same fibroblast markers was observed in a PDGFRA epicardial mutant at E18.5 (Figure 7A through 7D and Online Figure VIIB). These calculations were an overestimation of remaining fibroblasts as the nonepicardial-derived VSMC surrounding the coronary vasculature, the endocardium, and the epicardium also produced collagen (Figure 7A). To gain insights into the potential source of the cells in primary cardiac fibroblast cultures, we generated fibroblasts from Tie2Cre;R26RLacZ animals, in which all cells of endothelial origin should express β-galactosidase, and found that 21±7% (n=4 cultures) of the prolyl-4 hydroxylase or αSMA-expressing, adherent cells had an endothelial origin, as has been previously suggested.46

To determine whether a reduction in fibroblasts resulted in any extracellular matrix (ECM) defects, we examined hearts for levels of periostin (Postn), an extracellular matrix molecule secreted by cardiac fibroblasts.47 Periostin expression in PDGFREKO and PDGFRAEKO hearts had a marked reduction, whereas the periostin level in PDGFRIKO was unaffected (Figure 7F). We also investigated adult mice for generalized defects in matrix deposition. Focusing on perivascular regions, we used Masson Trichrome stain to identify collagen deposition from PDGFREKO and PDGFRAEKO hearts. Deletion of PDGFRA either individually or in combination with PDGFRI (PDGFRAEKO and PDGFRIKO, respectively) led to a reduction in collagen, while mice lacking PDGFRI (PDGFRAEKO) in epicardial cells and their derivatives were similar to controls (Figure 7G).

These results suggested that cardiac fibroblast development is disrupted in PDGFREKO and PDGFRAEKO hearts and that these epicardial-derived fibroblasts are required for matrix production in the developing heart. In conclusion, our data show a unique role for PDGF signaling in regulating epicardial EMT and fate specification of EPDCs.

Discussion

Since the discovery of the origin of cVSMC and cardiac fibroblasts 18 years ago, multiple signaling pathways have been identified that affect the formation, attachment, or EMT of the epicardium. However, few genes have been identified that are essential for cardiac fibroblast formation. Here, we have not only identified a unique requirement for PDGF receptor signaling in regulating epicardial EMT and EPDC formation, but also an essential role for PDGFRA, specifically in cardiac fibroblast formation.

Several growth factor signaling pathways have been implicated in the induction of EMT during development and various pathological states, and PDGF has been linked to the EMT process during cancer progression, organ
fibrosis, smooth muscle cell generation from the chicken proepicardium, and in the regenerating zebrafish heart. Some suggested mechanisms for PDGF's role in EMT include stabilization of β-catenin or induction of transcriptional activators of EMT, such as ZEB1/2 and Snai2. However, we do not observe these specific effects in epicardial cells after PDGF stimulation. We have identified the transcription factor Sox9 as a downstream target of PDGF-stimulated EMT. Our data show that Sox9 could rescue the EMT defect seen in PDGF receptor mutants and that Sox9 expression was upregulated on PDGF stimulation of epicardial cells. These data are consistent with the known role for Sox9 in avian neural crest cell EMT. Sox9 is a member of the SRY-related HMG-box family of transcription factors that is important for the development of many tissues and cell types. Cardiovascular defects have been reported in Sox9 mutants, but no epicardial phenotype has been described. Our data suggest that Sox9 could be an important component of PDGF receptor signaling during epicardial EMT, but further investigation is necessary to determine the mechanistic link between PDGF and Sox9.

Our data show that loss of both PDGF receptors led to defective EMT and failure to form any epicardial derivatives. Interestingly, individual deletion of the PDGF receptors also led to reduced epicardial EMT and a loss of only a subpopulation of EPDCs. There are 2 potential scenarios to explain when PDGF receptor function is required. In the first scenario, epicardial cells are heterogeneous, and each epicardial cell would only give rise to a specific lineage of...
PDGFRα is linked to a lineage-specific requirement of the PDGF receptors in controlling epicardial EMT. This process might regulate a lineage-specific EMT. Consistent with this possibility, experiments using limiting amounts of retrovirus to transduce the proepicardial organ revealed that tagged cells contributed only to the VSMC lineage. However, there have been no reports suggesting differential gene expression in the epicardium. For example, Tbx18, Tcf21, Raldh2, and both of the PDGF receptors seem to be uniformly expressed in the epicardium before EMT. A second possibility for PDGF function is that PDGF signaling by each receptor is redundant with regard to the EMT process, but after EMT, PDGFRα is expressed in fibroblast progenitors, and PDGFRβ is in cVSMC. In this scenario, the most likely role for PDGF receptor signal transduction is expansion and migration of the progenitor population. These unanswered questions require further investigation using temporal deletion to identify the window of epicardial fate specification.

The role of cardiac fibroblasts in heart pathogenesis is well appreciated, but the function of these cells during development is poorly understood. It has been proposed that cardiac fibroblasts perform a variety of essential duties during heart formation. These include stimulation of cardiomyocyte proliferation, isolation of the ventricular from the atrial conduction system, distribution of mechanical forces, and of course, deposition and degradation of ECM. Recent estimates are that cardiac fibroblasts comprise about 27% of the cells within the murine heart, but our data demonstrate that these cells are dispensable for heart development. Under nonpathological conditions, mice without epicardial derived fibroblasts lack adventitial collagen, but heart function is normal.

Because many cell populations have been proposed to contribute to fibrosis formation during pathological circumstances, there is the possibility that another source of fibroblasts fills the void. Proposed origins for this subpopulation include endothelial cells, fibrocytes, monocytes, and mural cells. A complete functional substitution by these cells in the absence of epicardial-derived fibroblasts is unlikely because mutant hearts that substitution by these cells in the absence of epicardial-derived fibroblasts lack adventitial collagen, but heart function is normal.

Comparison of PDGFRαEKO with PDGFRβEKO hearts did suggest a partial rescue of the ECM, presumably by the existing cVSMC, although the levels of matrix never appear to reach wild-type levels. While our initial examination suggests no major deficits in mutant animals, further studies are warranted to investigate cardiac homeostasis and other functional parameters such as conduction and stress response.

In summary, we demonstrate that cardiac fibroblast and cVSMC development is mediated by a combined role of the PDGF receptors in controlling epicardial EMT. This process appears to be linked to a lineage-specific requirement of the receptors. Specifically, PDGFRα is essential for cardiac fibroblast development. Finally, we establish a novel role for Sox9 as a critical downstream component of PDGF signaling in regulating epicardial EMT.

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**Disclosures**

None.

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Novelty and Significance

What Is Known?
- Cardiac fibroblasts and coronary vascular smooth muscle cells (cVSMC) are epicardial-derived cells (EPDC) that arise after an epithelial-to-mesenchymal transition (EMT).
- Platelet-derived growth factor receptor (PDGFR) \( \beta \) is required for coronary vascular smooth muscle cell development.
- PDGF (platelet-derived growth factor) signaling plays a role in coronary vessel remodeling during cardiac zebrafish regeneration.

What New Information Does This Article Contribute?
- PDGF receptors are required for epicardial EMT, and failure of this process leads to animals lacking EPDCs.
- Animals lacking EPDCs have no overt phenotype and are viable.
- PDGFR\( \alpha \) is the first receptor identified to be required for cardiac fibroblast formation, but PDGFR\( \alpha \) is dispensable for cVSMC development.
- The PDGF receptor genes are required in a lineage-specific manner for the formation of the 2 EPDC cell populations, cVSMC and cardiac fibroblasts.

Cardiac fibrosis is a major consequence of long-term cardiac disease, and the epicardium is the major source of resident cardiac fibroblasts that potentially contribute to this disease. Here, we report that PDGF receptor signaling is required for epicardial EMT. Expression of the transcription factor, Sox9, is reduced in epicardial cells lacking PDGF receptors, and expression of Sox9 rescues EMT in the absence of PDGF receptor signaling. We also report that disruption of the epicardial EMT process leads to the inability to generate cardiac fibroblasts and cVSMC. Additionally, the loss of PDGFR\( \alpha \) leads to a defect exclusively in cardiac fibroblast formation. This work is the first example of a lineage specific disruption of epicardial derivatives. Our findings show a previously unidentified role for PDGF receptor signaling in epicardial EMT and EPDC fate specification and provide a novel model to investigate the role of cardiac fibroblasts during embryogenesis as well as in the adult.
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Supplemental Experimental Procedures

EMT

Primary epicardial cultures were stimulated with 10% serum, 1-10ng/ml hTGFβ1 and 20ng/ml PDGFBB for 48 h. For immunocytochemistry, cultures were fixed in 4% paraformaldehyde for 10min. β-catenin (1:500, BD bioscience 610153) and phalloidin (1:200, Invitrogen A12379) were used for staining. For rescue experiments, cultures were treated with adenoviral-GFP, -LacZ (immunocytochemistry) or -Sox9 and treated as specified on the day of explant removal for 48h.

Microarray data can be accessed from the NIH GEO website (http://www.ncbi.nlm.nih.gov/geo/) under the accession number, GSE 27181.

Quantitative PCR

For qPCR analysis, RNA was isolated from three combined hearts for each sample or from two combined cultures for primary epicardial culture analysis using Trizol (Invitrogen). CDNA was generated with 1µg of RNA using SuperScript III (Invitrogen) and random hexamers. Gene expression was analyzed using standard qPCR methods with iTAQ Sybr Green master mix (Bio-Rad) on a CFX96 instrument (Bio-Rad). Each sample was run in triplicate and normalized to cyclophilin. Sequences for primers used in qPCR analyses can be found in Online Table I.

Immunohistochemistry

Information regarding immunohistochemistry is provided in Online Table II. All secondaries were from Molecular Probes and were used at a 1:500 dilution. Exact staining procedures are available upon request.

Flow Cytometry

Hearts from E13.5 or E16.5 PDGFRα<sup>GFP/+</sup> embryos or 6-week-old adults were dissected and conotruncal/valves were removed. Tissue was digested with 0.25% Trypsin-EDTA (Invitrogen) to generate a single cell suspension. PDGFRα<sup>GFP/+</sup> and PDGF receptor mutant adult hearts were digested as described for generating primary cardiac fibroblast cultures. Single cell suspensions were incubated with 1:100 dilution of PDGFRβ-PE (eBioscience, 12-1402-81). For detecting epicardially derived cardiac fibroblasts, single cell suspensions from control and PDGF receptor mutant adult hearts were incubated with a 1:100 dilution of Thy1-PE (Invitrogen, MCD9004) and 1:100 CD31-APC (eBioscience, 17-0311-80). All samples were analyzed on a BD FACSCalibur.

Cardiac fibroblast quantification

Masson-trichrome staining was performed on 8-10 week old adult hearts fixed in 10% buffered formalin and paraffin embedded. Hearts were sectioned coronally into 7µm thick sections to generate a four-chamber view and staining was done according to manufacturer’s protocol (Thermo scientific, MA). Quantification was conducted by imaging a minimum of 7 different vessels from non-consecutive regions from the lateral ventricles. Collagen deposition was quantified as described elsewhere <sup>1</sup> using ImageJ (NIH) and normalized to the vessel circumference.
Collagen 1a1 in situ and P4hb IHC was quantified by measuring the area of positive staining excluding perivascular, endocardial, and epicardial expression using ImageJ (NIH). A minimum of 2 sections per heart was quantified to generate an average for the indicated genotypes.

Cell culture and isolation

Primary epicardial cells were cultured from E12.5 hearts as published previously. Cultures used for immunocytochemistry were plated on glass coverslips coated with 5ug/cm² Collagen IV (R&D Systems) and incubated for four days prior to removing explant. Primary cardiac fibroblasts were isolated from 3-4 week hearts by first removing the atria, aorta, and cardiac valves. The tissue was subsequently minced with forceps and incubated with 0.2U/ml of Blendzyme 3 (Roche) in Earl’s buffer (140mM NaCl, 8mM KCl, 1.8mM CaCl₂, 0.9mM MgCl₂, 25mM HEPES, pH 7.4) for 30min at 37°C. Cells were washed in PBS and plated overnight in DMEM with 10% serum. Cells were harvested, plated overnight, fixed in 0.2% glutaraldehyde for 5 min., and stained for β-galactosidase activity. Cells were quantified by imaging a minimum of 3 20x fields of view and counting the number of β-galactosidase+ cells/total of cells.

Adenoviral production

AdLacZ and AdGFP were kindly provided by Robert Gerrard (UTSW). AdSox9 was generated from a full-length cDNA (Open Biosystems, 5320371) and cloned into (pAd/CMV/V5-DEST, Invitrogen).

Western Blotting

Protein was isolated from P1 hearts. Positive control protein was generated from differentiated MC3T3-E1 cells. Western blot membranes were incubated with anti-periostin (1:500, Santa Cruz SC-49480) or anti-β tubulin (1:1000, BD 556321) overnight at 4°C, washed and probed with anti-goat HRP (periostin, 1:1000, sigma) or anti-Mouse IgM (β tubulin, 1:3000, Zymed).

Migration

The ex vivo migration assay was performed as described previously.

Imaging

The following equipment was used for imaging: Fluorescent imaging (Zeis Axiovert 200 with a Hamamatsu ORCA-ER camera), confocal imaging (LSM510META) (grant NIH1-S10-RR019406-01), color imaging (Zeis Axiovert 200 with an Olympus DP71 camera), and whole mount imaging (Nikon SMZ1000 with an Olympus DP71 camera). Images and figures were edited and created in Photoshop CS4. Figure 4A hearts were cropped to avoid confusion with Sox9+ cells in the body wall.

Statistics

All statistical calculations were done using Prism 5 (GraphPad). Student t-test, one-way and two-way ANOVA tests were done where appropriate and subsequently analyzed using Tukey and Bonferroni post tests for determining significance.

Proliferation index

Proliferation was measured as described previously with a 1 hour BrdU labeling time.
In situ hybridization

*In situ* hybridization was done as described previously. Digoxigenin labeled Tbx18 and Tcf21 probes were synthesized from the plasmids as previously described and were kind gifts from Sylvia Evans (UCSD) and Ondine Cleaver (UTSW), respectively. Plasmid template for Col1a1 probe generation was a kind gift from Gerard Karsenty (Columbia). PDGFRα was synthesized as previously described.
Online Figure I. Epicardial specification, proliferation and apoptosis

(A) Whole mount in situ hybridization for epicardial transcription factors as indicated. (B) IHC for Podoplanin, a protein expressed by developing epicardial cells. Myo-myocardium and epi- epicardium. (C-D) Quantification of BrdU incorporation and activated (cleaved) caspase 3 staining. (*) p<0.001 (SEM – subepicardial mesenchyme)
Online Figure II. PDGFR$^{EKO}$ epicardial cells fail to migrate
(A) Vimentin IHC was used to examine epicardial cell migration into subepicardial mesenchyme. Note that endothelial cells are also vimentin positive. Arrowheads point to subepicardial mesenchyme. Dashed lines delineate border between myocardium and subepicardial zone, myo-myocardium and epi-epicardium. (B) E15.5 hearts were stained for WT1 and quantified for WT1$^+$ cells/FOV within the myocardium. N values are indicated in parentheses. (*) p<0.001
Online Figure III. EMT morphology

(A) Brightfield images of control and PDGFR<sup>EKO</sup> epicardial cultures stimulated with hTGFβ<sub>1</sub> and PDGFBB. (B) Bright field images of primary epicardial cultures of the indicated genotypes transduced with AdLacZ or AdSox9 in the presence of vehicle or stimulation (hTGFβ<sub>1</sub> and PDGFBB). (C) qPCR analysis of primary epicardial cultures showed loss of PDGF receptor expression in the presence of Cre. (*) p<0.001 (compared to stimulated control), (#) p<0.001 (compared to vehicle control). (D-F) qPCR analysis demonstrated that AdGFP transduction did not alter primary epicardial culture responses to stimulation. Data are represented as mean ± SD. N values are indicated in parentheses.
Online Figure IV. Sox9+ cells are derived from the epicardium and numbers vary according to genotype (A) WT1+Cre/+ R26R<sup>YFP</sup> lineage traced hearts. Embryos were induced with tamoxifen at E12.5 and costained with Sox9 and GFP antibodies at E14.5. Arrows show overlap of YFP<sup>+</sup> and Sox9<sup>+</sup> cells. Note this embryo had less efficient cre induction, as few YFP<sup>+</sup> cells are present in the epicardium. When Cre induction (at E12.5) is efficient, a high percentage of labeled cells remain in the epicardium (See manuscript). (B) Sox9 expression in the indicated genotypes. We include this image to illustrate that in a wild type (no mutant alleles for the PDGF receptors), we observe even more Sox9<sup>+</sup> cells than in our controls. Our controls often contained two recombined PDGF receptor alleles (one PDGFRα and one PDGFRβ, designated as Δ) due to Gata5Cre recombination in the male germline.
Online Figure V. PDGF receptor expression during development
(A) Flow cytometric analysis of PDGF receptor expression on cells isolated from embryonic and postnatal hearts with endocardial cushion/valves removal. Antibody detection was used for PDGFRβ while GFP fluorescence was used for PDGFRα expression. (n=1 at E13.5 and n=2 at E16.5 and adult).
Online Figure VI. Cardiac function in PDGFR^{EKO} animals
(A-B) Heart weight / body weight and heart weight / tibia length ratios in 8- to 12-week old mice of the indicated genotypes. (C) Fractional shortening of 8- to 12-week old mice of the indicated genotypes as measured by echocardiography. N values are indicated in parentheses.
Online Figure VII. Collagen 1a1 expression during postnatal development
(A) Coll1a1 and PDGFRα in situ hybridization of left ventricles at the indicated ages. (B) PDGFRα in situ hybridization of control and PDGFRα mutant hearts. Timecourse was completed using wild type animals of a mixed background.
### Online Table I: Primers used for qPCR analysis

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Online Table II: Antibodies

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Supplemental References
