Focal Adhesion Kinase Regulates Smooth Muscle Cell Recruitment to the Developing Vasculature

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Objective—The investment of newly formed endothelial cell tubes with differentiated smooth muscle cells (SMC) is critical for appropriate vessel formation, but the underlying mechanisms remain unknown. We previously showed that depletion of focal adhesion kinase (FAK) in the *nkx2.5* expression domain led to aberrant outflow tract (OFT) morphogenesis and strove herein to determine the cell types and mechanisms involved.

Methods and Results—We crossed fak^{loxp} targeted mice with available Cre drivers to deplete FAK in OFT SMC (FAK^{wnt} and FAK^{nk}) or coronary SMC (FAK^{cSMC}). In each case, depletion of FAK led to defective vasculogenesis that was incompatible with postnatal life. Immunohistochemical analysis of the mutant vascular structures revealed that FAK was not required for progenitor cell proliferation, survival, or differentiation into SMC but was necessary for subsequent SMC recruitment to developing vasculature. Using a novel FAK-null SMC culture model, we found that depletion of FAK did not influence SMC growth or survival, but blocked directional SMC motility and invasion toward the potent endothelial-derived chemokine, platelet-derived growth factor PDGFBB. FAK depletion resulted in unstable lamellipodial protrusions due to defective spatial-temporal activation of the small GTPase, Rac-1, and lack of Rac1-dependent recruitment of cortactin (an actin stabilizing protein) to the leading edge. Moreover, FAK null SMC exhibited a significant reduction in stimulated extracellular matrix degradation.

Conclusion—FAK drives PDGFBB-stimulated SMC chemotaxis/invasion and is essential for SMC to appropriately populate the aorticopulmonary septum and the coronary vascular plexus. **(*Arterioscler Thromb Vasc Biol*. 2011;31:2193-2202.)**

Key Words: biology developmental ■ extracellular matrix ■ morphogenesis ■ vascular biology

The investment of newly formed endothelial cell tubes with differentiated smooth muscle cells (SMC) is a very important process during vessel formation and requires intricate regulation of SMC specification, motility, growth, and differentiation. Failure of SMC recruitment to and migration along developing vessels can lead to vascular instability and regression, an event that is likely due in part to the ability of these cells to secrete and organize extracellular matrix-containing basement membranes and elastin. Moreover, mature medial SMC express high levels of contractile genes (ie, myosin heavy chain, α-smooth muscle actin (α-SMA), SM22 among others) and their presence is essential for maintaining vessel tone and for providing dynamic control of blood pressure (see[1] for review).

Although the precise signaling pathways have yet to be identified, a number of major extrinsic factors have been shown to regulate SMC recruitment and function. TGF-β, which promotes SMC differentiation,[2–4] and platelet-derived growth factor (PDGF), which promotes SMC growth and motility, are both important extrinsic regulators of SMC phenotype and genetic ablation of receptors for these genes resulted in defective outflow tract (OFT) or coronary vascular morphogenesis.[5–7] Extensive evidence indicates that extracellular matrix signaling is also an important regulator of SMC growth and differentiation as deletion of either fibronectin, the α5 integrin FN receptor, or focal adhesion kinase (FAK) (the kinase that mediates α5-dependent signaling) each results in extraembryonic and embryonic vessel defects leading to lethality in the mouse from E8.5 to E10.[8–10]

Although a direct role for FAK in vascular smooth muscle growth and development has yet to be examined, our laboratory recently showed that depletion of FAK from *nkx2.5*-expressing precursors led to perinatal lethality resulting from a profound subaortic ventricular septal defect accompanied (in some neonates) by malalignment of the OFT including double-outlet right ventricle and persistent truncus arteriosus

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Mouse Line Abbreviations: FAK^{nk} $fak^{flox/-}$ $nkx2.5^{Cre/+}$, FAK^{wnt} $fak^{flox/-}$ $wnt\text{-}1^{Cre/+}$/$Rosa26R^{lacZ/wt}$, FAK^{cSMC} $fak^{flox/-}$ $wt\text{-}1^{Cre/+}$/$Rosa26R^{lacZ/wt}$.

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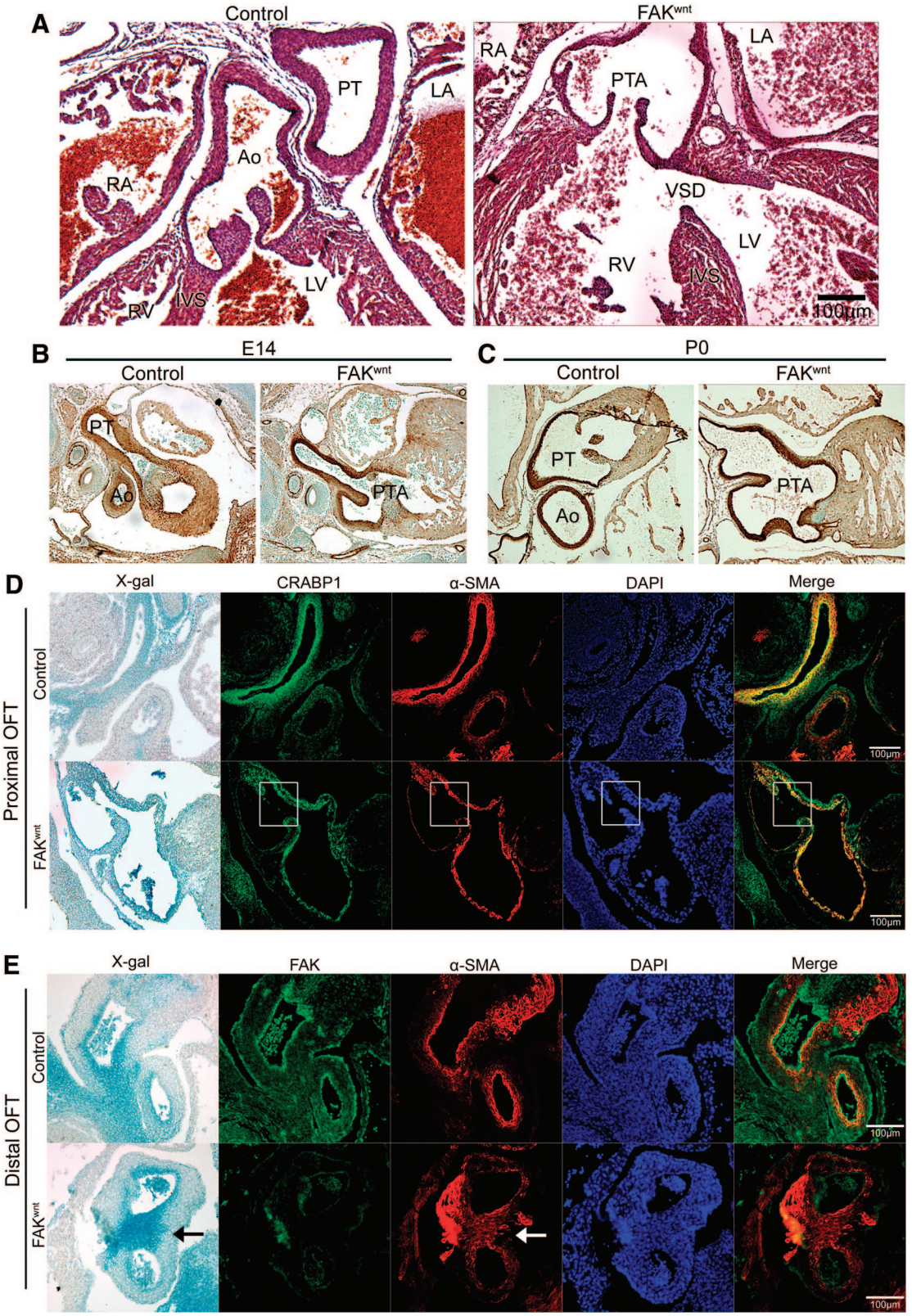

Figure 1. Focal adhesion kinase (FAK) deletion impairs recruitment of cardiac neural crest-derived smooth muscle cells (SMC) to the developing aorta and pulmonary artery. **A**, H&E staining of genetic control and FAK^{wnt} mice at postnatal day 0 (P0). FAK^{wnt} outflow tract (OFT) shows persistent truncus arteriosus (PTA) and ventricular septal defect (VSD). LA indicates left atrium; RA, right atrium; Ao, aorta; PT, pulmonary trunk; IVS, intraventricular septum. **B**, **C**, SM22 immunostaining (brown) of embryonic day 14 (E14; B) or P0 (C) genetic control and FAK^{wnt} OFT revealed PTA without defect in SMC differentiation. **D**, Proximal OFT of genetic control and FAK^{wnt} hearts at E 12 reveals colocalization of α-SMA (red) with the cardiac neural crest lineage marker CRABP1 (green), indicating that the SMC covering the aorta and pulmonary artery were derived from cardiac neural crest cells. X-gal staining demarcates *wnt-1*–derived cardiac neural crest in the Rosa26R^{LacZ}–positive embryos. Nuclei (blue) were stained with DAPI. Note the lack of SMC coverage

(PTA).[11] Thus, conditional inactivation of FAK in *nkx*2.5-expressing cells phenocopies the most common congenital heart disease that is also a subset of abnormalities associated with Tetralogy of Fallot and the DiGeorge Syndrome.[12,13] However, whether these defects arose from the inability of *nkx2.5* precursor cells to differentiate into SMC or from a specific defect in *nkx2.5*-derived SMC remained unclear.

The aim of the present study was to identify the critical FAK-dependent functions during vasculogenesis. Because both *wnt-1*- and *nkx2.5*-expressing cells contribute to development of conotruncal septal SMC that divide the OFT into the aorta and pulmonary artery,[13,14] we explored the effects of FAK depletion in both origins using available Cre lines. We show FAK deletion (by homologous recombination) in either embryological origin results in OFT defects including PTA. Moreover, targeted depletion of FAK in these SMC precursors did not affect progenitor cell proliferation, survival, or differentiation but resulted in lack of appropriate SMC coverage of the developing vasculature. Similarly, depletion of FAK in a third SMC precursor origin, the *wt1*-expressing epicardial cells that provide SMC that line coronary vessels, did not affect SMC specification but led to aberrant recruitment of SMC to the developing coronary vasculature. Collectively, these studies indicate that FAK is essential for a SMC autonomous function during vascular remodeling. Our mechanistic studies in FAK null SMC, revealed that FAK does not influence SMC growth or survival but functions to promote directional SMC chemotaxis/invasion toward the potent endothelial-derived chemokine, PDGF.

Methods

Experimental Animals

$fak^{+/-}$ mice[15] were bred to existing Cre lines including cardiac neural crest (CNC)-specific *wnt-1*^{Cre} mice obtained from Andrew McMahon[14]; primary and secondary heart field-specific *nkx2.5*^{Cre} mice obtained from Robert Schwartz[16]; and epicardial-specific *wt1*^{Cre} mice obtained from John Burch.[17] The resulting $fak^{+/-}$/Cre$^{\pm}$ mice were bred with $fak^{flox/flox}$/Rosa26R$^{lacZ/lacZ}$ mice to obtain $fak^{flox/-}$ Cre$^{+/-}$/Rosa26R$^{lacZ/wt}$ (experimental) or genetic control mice ($fak^{flox/-}$/Rosa26R$^{lacZ/wt}$ without Cre and $fak^{flox/+}$ with or without Cre). Mice were housed in the University of North Carolina Animal Care Facility, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and all experimental procedures were approved by the University of North Carolina Animal Care and Use Committee.

Statistical Analysis

All quantitative data represent at least 3 separate experiments and are presented as mean±SEM. Means were compared by 2-tailed Student *t* test. $P<0.05$ was considered statistically significant.

See Supplemental Data Section for additional experimental procedures, available at http://atvb.ahajournals.org.

Results

FAK Deletion Impairs Recruitment of *nkx*2.5- and *wnt-1*–Derived SMC to the Developing Aorta and Pulmonary Artery

We previously showed that conditional deletion of FAK in *nkx2.5*-expressing cells (FAK^{nk}) resulted in malalignment of the OFT and incomplete aorticopulmonary septum formation in a percentage of neonates.[11] Because *nkx2.5*-derived SMC contribute to the formation of the aortic root and septum, we hypothesized that this phenotype may have resulted from a SMC defect in FAK-depleted cells. To further examine FAK's role in OFT development, we used the *wnt-1*^{Cre} line to deplete FAK from the CNC-derived SMC that also make a major contribution to this process.[14] These well characterized mice generate high-efficiency recombination around E9.5 in the CNC-derived cells populating the truncus arteriosis and pharyngeal arch arteries (3, 4, and 6) and in the CNC-derived cells invading the conotruncal cushions and the aorticopulmonary septum by E11.5.[14] Although $fak^{flox/-}$wnt-1$^{Cre/+}$/Rosa26R$^{lacZ/wt}$ mice (herein referred to as FAK^{wnt}) were present at the appropriate Mendelian ratio at P0 (Supplemental Table I), all FAK^{wnt} mice died within a few days after birth. Gross morphology of the cardiac OFT revealed aberrant septation and branching of the major outflow vessels in all FAK^{wnt} mice with the majority (95%; N=42/44) exhibiting PTA (Figure 1A). Thus, our data indicate that FAK activity in *nkx2.5*- and *wnt-1*–derived cells is required for appropriate septation of the truncus arteriosis.

Because the aortic arch defects observed in CNC-targeted Notch$^{-/-}$ mice were associated with impaired SMC differentiation within the truncus,[18] we tested whether FAK depletion affected this process in our models. Expression of α-SMA in the pharyngeal arch region at E11 was similar between control and FAK^{wnt} mice (Supplemental Figure IA). The unseptated ascending OFT in FAK^{wnt} mice contained layers of SM22-positive medial SMC that were comparable to littermate controls from E14 onwards (Figure 1B and 1C). SMC layers and differentiation in the carotid arteries also appeared to be normal in FAK^{wnt} mice (Supplemental Figure IB). These mice were bred to contain the Rosa26R^{LacZ} allele to track progenitors of the Cre-expressing cells, and the neural crest origin of carotids was confirmed by β-galactosidase positive staining (Figure S1C). In addition, as shown in Supplemental Figure ID, SMC investment of coronary SMC (that were not targeted for FAK depletion) was also similar between genetic control and FAK^{wnt} hearts, indicating that global changes in flow or hemodynamic properties do not account for the SMC investment phenotypes observed in the FAK^{wnt} OFTs.

Aorticopulmonary septation occurs between E11.5 and E12.5 when a wedge of CNC-derived SMC partitions the aortic sac into distinct aortic and pulmonary channels.[19] To more closely evaluate this process in FAK^{wnt} mice, serial sections were costained for α-SMA and CNC lineage markers (CRABP1 or expression of the Rosa26R^{LacZ} allele). As shown in Figure 1D, the SMC lining the pulmonary artery and aorta at the level of the proximal OFT are mainly derived from CNC in control and FAK^{wnt} mice. However, a selective lack of CNC (and CNC-derived SMC) was apparent in the aortic wall juxtaposed to pulmonary artery in the FAK^{wnt}

Figure 1 (Continued). (highlighted in white boxes) in the inner walls of the aorta and pulmonary artery in FAK^{wnt} mice. **E**, Distal OFT at E 12 stained with X-gal (blue in bright field), FAK (green), and α-SMA (red) reveals FAK-depleted cardiac neural crest differentiate into SMC (arrows) but fail to appropriately populate the conus. Nuclei (blue) were stained with DAPI. Scale bar=100 μm. LV indicates left ventricle; RV, right ventricle.

mice (Figure 1D; highlighted in box). FAK deficient CNC cells populated the conotruncal region dorsal to the PTA and properly differentiated into SMC (Figure 1E). However, in FAK^{wnt} mice the SMC in the dorsal conus were more centrally located (Figure 1E; arrow) and did not surround the developing vessels as completely in control mice.

We previously showed that FAK deficiency did not alter proliferation or survival within the affected OFTs of FAK^{nk} mice.[11] Similarly, there was no significant difference in levels of cell death in the OFT of E13.5 FAK^{wnt} mice in comparison to controls (27.8±4.3 versus 29.3±7.4 per 0.5 mm^2, respectively; N=5/5). The numbers of BrdU-labeled cells in the affected OFT region of FAK^{wnt} embryos also did not differ significantly from those in the genetic controls (45.8 +/−3.7 versus 43.6 +/−4.8 per 0.5 mm^2, respectively; N=4/4). In support of a lack of major effect on the proliferation and survival of CNC-derived cells, the more distal CNC-derived structures such as the aforementioned carotid arteries (Supplemental Figure IB and IC) and other neural crest-derived structures including the thymic lobes, thyroid, tongue, and ear appeared to be unaffected by FAK deletion (data not shown). Close evaluation of the cranial cartilage and bones did reveal a modest mandibular truncation in 3/6 FAK^{wnt} neonates, but the crown-rump length and maxilla formation were normal (Supplemental Figure IE). Collectively, these studies reveal that CNC functions, population of the developing OFT, and differentiation of *wnt-1* lineages into SMC occur normally in FAK^{wnt} mutants. However, in these mice and those in which FAK was depleted in *nkx2.5*-derived cells, FAK null SMC fail to appropriately invest the inner walls of the developing aorta and pulmonary trunk, indicating that FAK may be necessary for directional SMC movement within the conotruncus.

FAK Deletion Impairs Recruitment of Epicardial-Derived SMC to the Coronary Plexus

To provide additional in vivo evidence for our hypothesis, we tested the effects of FAK depletion on the recruitment of epicardial-derived SMC to the intramyocardial endothelial plexus, a process also dependent on directional motility.[7] To this end, we intercrossed our FAK$^{flox/flox}$/Rosa26R$^{lacZ/lacZ}$ mice to an available *Wilm's Tumor-1*^{Cre} line (*wt-1,* hereafter referred to as FAK^{cSMC} so as to not be confused with FAK^{wnt}) that induces strong and uniform recombination in the proepicardium by E9.5 and is restricted to the epicardium from E10.5 onward.[17,20] Previous studies revealed that *wt1*-derived cells give rise to the SMC that invest the coronary arteries and veins,[7,20] and we found similar results on analysis of β-galactosidase expression in P0 *wt1*^{Cre}/Rosa26R^{LacZ} hearts (Supplemental Figure II). We observed Mendelian distribution of FAK^{cSMC} at P0 (Supplemental Table I), but all FAK^{cSMC} mice died within a few days after birth by still to be determined mechanisms.

Importantly, immunohistochemical assessment of α-SMA positive cells in P0 hearts, revealed a striking and consistent lack of SMC coverage of the coronary endothelial plexus (Figure 2). All large diameter vessels in FAK^{cSMC} hearts exhibited a thin noncontinuous layer of SMC in comparison to comparable genetic control vessels (Figure 2B, arrowheads), although most of the smaller arterioles exhibited a

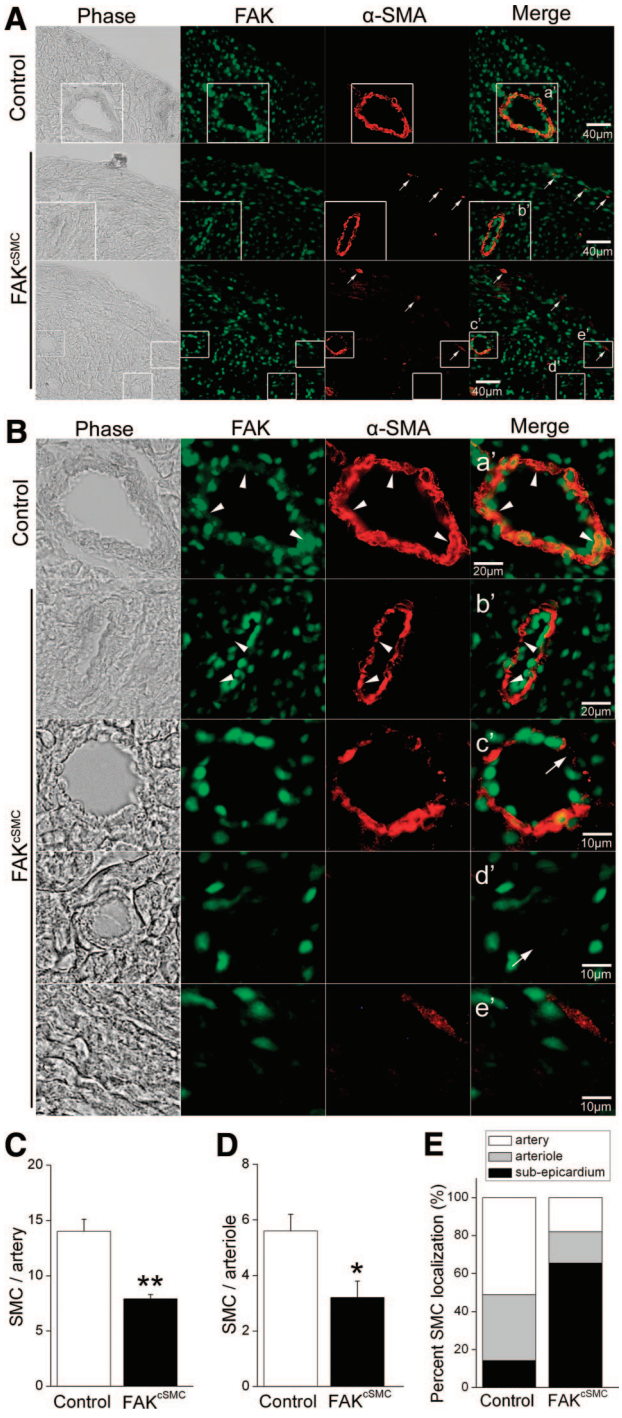

Figure 2. Focal adhesion kinase (FAK) deletion impairs recruitment of epicardial-derived smooth muscle cells (SMC) to the coronary vessels. **A**, P0 genetic control and FAK^{cSMC} heart stained with FAK (green) and α-SMA (red). Phase image is shown on left. Subepicardial α-SMA-stained cells (arrows) are present in FAK^{cSMC} but not genetic control hearts. Scale bar=40 μm. Boxed regions (a'-e') in A are shown at higher magnification in B. **B**, FAK^{cSMC} hearts exhibited reduced presence of SMC lining the coronary vasculature, including large vessels (arrowheads) and small arterioles (arrows), in comparison to genetic control vessels. FAK deletion was confirmed by absence of FAK staining in SMC from FAK^{cSMC} hearts (arrowheads). Scale bar=20 μm or 10 μm. **C–E**, Numbers of SMC lining arteries (diameter >65 μm), arterioles (diameter <65 μm as previously described[45]), or localized in the subepicardium were quantified using Image J. *P<0.05; **P<0.01.

complete lack of associated SMC (Figure 2B, arrows). Quantification of SMC numbers revealed significantly fewer SMC associated with both arteries and arterioles in FAK^{cSMC} hearts relative to genetic controls (Figure 2C,D). In contrast, significantly higher numbers of α-SMA positive cells were observed in the subepicardial space in FAK^{cSMC}, compared to genetic control hearts (Figure 2A, arrows, Figure 2E), indicating that the FAK null cells efficiently delaminated from the epicardium and differentiated into SMC, but failed to migrate into the myocardium. Importantly, a reduction in platelet/endothelial cell adhesion molecule labeled coronary vasculature was not observed in the FAK^{cSMC} hearts (Figure 3), indicating appropriate formation of the primary plexus. This conclusion is supported by the finding that these hearts did not exhibit signs of hypoplastic growth or noncompaction that are associated with defects in endothelial plexus function (Supplemental Figure IIIA and IIIB).[21] As well, no defects in the formation or SMC investment in carotid arteries (not targeted for FAK depletion) were observed in the FAK^{cSMC} neonates, indicating a lack of effect on global hemodynamics (Supplemental Figure IIIC).

We next performed additional studies to confirm that FAK activity was not required for the proliferation or survival of SMC progenitors, or their differentiation into coronary SMC. As shown in Supplemental Figure IVB, there was no statistical difference in the rates of cell proliferation or apoptosis in β-gal labeled *wt-1*-derived cells within FAK^{cSMC} hearts compared to genetic controls at either E15.5 or P0. Interestingly, by E15.5, many β-gal labeled cells in genetic control hearts had moved into the subepicardial zone, whereas most of the β-gal labeled cells in E15.5 FAK^{cSMC} hearts remained associated with the epicardium (Supplemental Figure IVA), corroborating our previous findings in the P0 hearts that suggested impaired directional motility of the FAK-depleted SMC (Figure 2B and 2E). We also analyzed SMC differentiation marker gene expression in quail proepicardial organ explants treated with adenoviruses that express GFP or the FAK inhibitor, GFP-FRNK. As shown in Supplemental Figure V, expression of FRNK attenuated the outward movement of the epicardial explants but did not affect the induction of SMC marker gene expression in these cultures. These studies strongly suggest that the SMC investment defect observed in FAK^{cSMC} coronary vessels did not result from a block in the proliferation, survival, or differentiation of *wt-1* progenitors but rather from a lack of SMC recruitment to the endothelial plexus.

FAK Deletion Impairs SMC Chemotaxis but not Proliferation or Survival

To confirm that FAK plays a significant role in the regulation of SMC chemotaxis, we established a conditional FAK null SMC culture model using cells isolated from *fak*$^{flox/flox}$ mice. As shown in Figure 4A, treatment of *fak*$^{flox/flox}$ SMC with Cre adenovirus (but not LacZ) resulted in a significant reduction of FAK protein. In excellent agreement with our in vivo studies, FAK deletion had no effect on SMC differentiation marker gene expression (Figure 4A) and, importantly, did not alter the expression of FRNK or the FAK homologue, Pyk2 (Supplemental Figure VIA). Moreover, continuously adherent FAK null cells revealed no major differences in focal adhesion or actin filament organization (Supplemental Figure VIB).

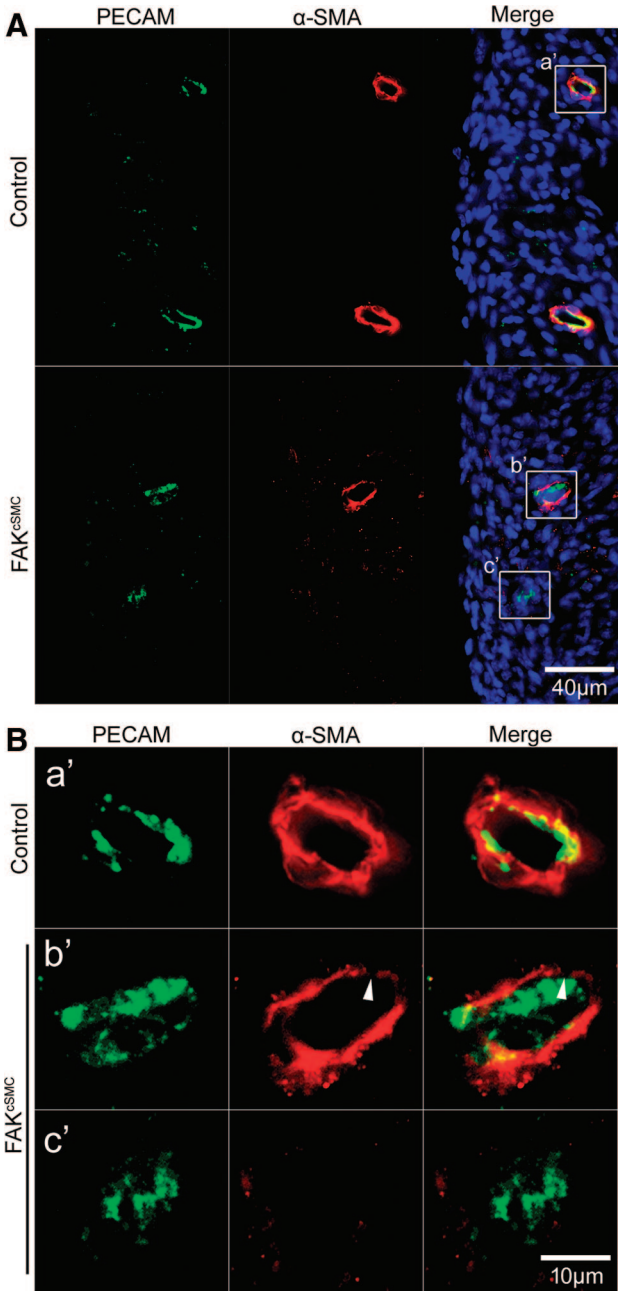

Figure 3. Endothelial plexus formation in focal adhesion kinase (FAK)^{cSMC} hearts. P0 genetic control and FAK^{cSMC} hearts stained with endothelial marker platelet/endothelial cell adhesion molecule (PECAM) (green) and α-SMA (red) showing normal endothelial plexus formation with reduced smooth muscle cell coverage (arrowhead) of the coronary endothelial plexus. Nuclei (blue) were stained with DAPI. Boxed regions (a'-c') in **A** are shown at higher magnification in **B**. Scale bar=40 μm (A) and 10 μm (B), respectively.

Because the strongly chemotactic PDGFs are highly expressed in the conotruncus during OFT and coronary vessel morphogenesis, and have been implicated in guiding cells during these critical processes,[5,22] we hypothesized that the cause of the PTA (in FAK^{wnt} and FAK^{nk} mice) and the impaired coronary vessel formation (in FAK^{cSMC} mice) was

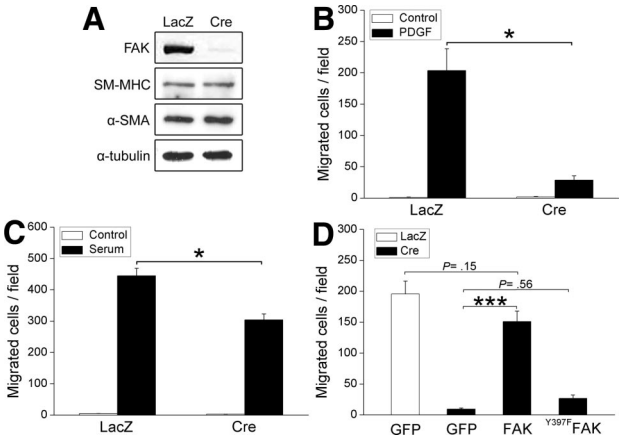

Figure 4. Focal adhesion kinase (FAK) deletion impairs smooth muscle cell chemotaxis/invasion toward platelet-derived growth factor (PDGF) and serum. **A**, *fak*$^{flox/flox}$ SMC were infected with either LacZ or Cre adenovirus for 72 hours and cell lysates were immunoblotted with antibodies for smooth muscle differentiation markers (smooth muscle myosin heavy chain (SM-MHC) and α-SMA). Alpha-tubulin was used as a loading control. Data are representative of at least 4 experiments. **B–D**, Cells treated as above were plated on fibronectin-coated inserts (10 μg/mL; BioCoat) in serum-free media using either PDGF-BB (20 ng/mL; **B**) or 10% serum (**C**) as the chemoattractant. **D**, LacZ- or Cre-infected cells were cotransfected with GFP and either Flag vector control, Flag-FAK, or the inactive mutant Flag-^{Y397F}FAK. Chemotaxis toward PDGF-BB was assessed 24 hours after transfection. Wild-type FAK but not ^{Y397F}FAK restored chemotaxis toward PDGF in the Cre expressing cells. Results in **B–D** are mean±SEM of cells counted in 4 fields from 4 independent experiments. *$P<0.05$; ***$P<0.001$.

due to a defective SMC migratory response to this chemokine. We found that PDGF treatment induced robust FAK activation at the leading edge of SMC (Figure S6C) and that FAK was necessary for PDGF-stimulated SMC chemotaxis as assessed using fibronectin-coated transwells (Figure 4B). Chemotaxis toward serum was also inhibited but this effect was much more modest (Figure 4C). Importantly, ectopic expression of wildtype FAK but not phosphorylation-deficient FAK (^{Y397F}FAK) restored PDGF-stimulated motility in FAK-deficient cells (Figure 4D). Confirming our in vivo results, we detected no significant difference in cell proliferation between control and FAK null SMC grown in 10% serum (data not shown), under serum-free conditions, or following treatment with PDGF or EGF (Supplemental Figure VID). Accordingly, PDGF receptor activation (as assessed by staining and Western analysis for pY716PDGFRβ; data not shown), PDGF-stimulated ERK activity (Supplemental Figure VIE) and cyclin D protein levels (Supplemental Figure VIF) were similar in Cre- and LacZ-treated cells, indicating that FAK was not required for activation of the major mitogenic Ras/ERK signaling pathway. We also observed no significant difference in cell survival in FAK-depleted SMC as measured by caspase 3/7 activity (data not shown). As well, PDGF-stimulated activation of the prosurvival kinase, AKT was unchanged (data not shown). Collectively, these data indicate that defective aorticopulmonary septation and coronary vasculogenesis observed in our conditional FAK mutants was likely due to a specific defect in PDGF-dependent SMC chemotaxis.

FAK Increases Lamellipodial Stability Through Rac1-Dependent Recruitment of Cortactin

Because PDGF-dependent motility involves rapid remodeling of the plasma membrane and underlying actin cytoskeleton, we next explored whether FAK-depleted SMC exhibited a biomechanical defect in one of these processes. To this end, we performed time-lapse imaging of Cre- and LacZ-treated *fak*$^{flox/flox}$ SMC following PDGF treatment. In control cells, PDGF stimulated extensive membrane ruffling as early as 2.5 minutes that culminated in formation of a dominant, stable leading edge lamellipodium by 15 to 20 minutes (Figure 5A, left). In contrast, PDGF treated FAK null SMC remained unpolarized and rarely formed a distinctive leading edge (Figure 5A, right). Kymographic analysis of lamellipodia dynamics indicated that this difference was mainly due to decreased lamellipodia persistence in FAK null SMC (Figure 5B-5D) and that this defect culminated in a significant decrease in leading edge displacement (Figure 5E) and cell speed in 2-D (Figure 5F).

PDGF-stimulated motility is dependent on Rac1, a small GTPase that controls cell protrusion and lamellipodial dynamics. To test whether FAK is necessary for Rac1 activation, we first analyzed total Rac1 activation in response to PDGF using the standard GST-PBD pull-down assay. As shown in Supplemental Figure VIG, PDGF robustly and transiently activated Rac1 with peak activity by 2.5 minutes and, somewhat surprisingly, this response was not affected by depletion of FAK. Thus, global activation of Rac1 is FAK-independent. Because recent studies indicate that PDGF induces leading edge localized activation of Rac1 via induction of new integrin-extracellular matrix connections,[23] we explored the idea that FAK might be necessary for restricting localized Rac1 activity to the leading edge. Immunofluorescent staining with a Rac1 antibody revealed that PDGF treatment of wildtype and FAK-deficient SMC led to rapid and equivalent accumulation of Rac1 at dorsal ruffles that peaked at 2.5 minutes (Figure 5G top, 5H). By 15 minutes following PDGF-treatment, restricted enrichment of Rac1 at the leading edge was observed in wildtype SMC, but this response was perturbed in FAK null SMC, which exhibited random distribution of membrane-associated Rac1 (Figure 5G bottom, 5I).

To confirm a role for FAK in localized Rac1 activation, we next examined the spatiotemporal distribution of cortactin, an actin binding protein that is recruited to the cell periphery in a Rac1-dependent fashion.[24] Immunofluorescent staining showed that recruitment of cortactin to leading edge-lamellipodia (Figure 5J bottom, 5L), but not dorsal ruffles (Figure 5J top, 5K) was significantly reduced in FAK null SMC, indicating that FAK is critical for this aspect of PDGF-stimulated cytoskeletal remodeling. These data are consistent with the finding that active FAK accumulates at the leading edge of cells following PDGF treatment (Supplemental Figure VIC). Importantly, either blocking Rac1 activity with the pharmacological inhibitor NSC23766 (10 μmol/L, Calbiochem) or treatment with cortactin siRNAs mirrored the effects of depleting FAK on PDGF-stimulated SMC membrane dynamics and chemotaxis (Supplemental Figure VII). In sum, these studies indicate that FAK plays a

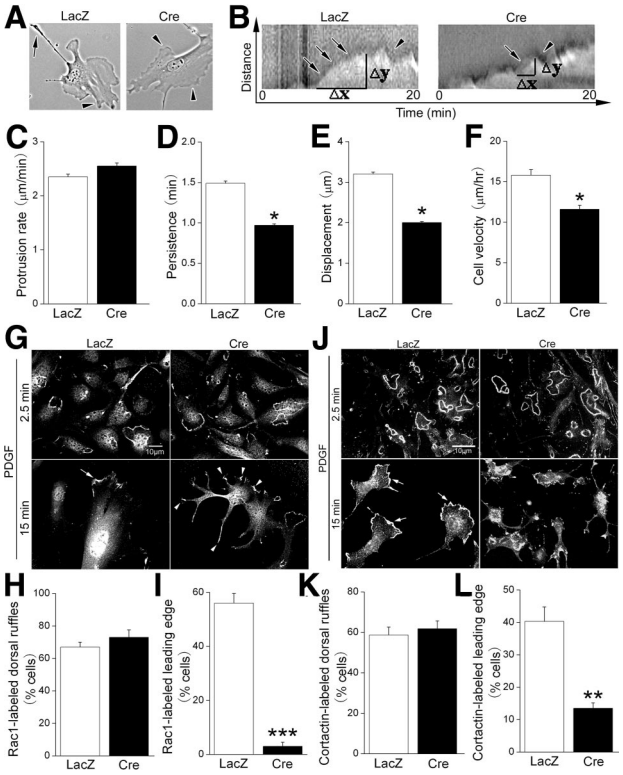

Figure 5. Focal adhesion kinase (FAK) regulates Rac1-dependent cortactin recruitment and lamellipodial stability. LacZ- or Cre-infected *fak*$^{flox/flox}$ SMC were treated with 20 ng/mL platelet-derived growth factor (PDGF)-BB and immediately imaged at 5-second intervals for 60 minutes. **A**, Representative images of cells 20 minutes following PDGF treatment. Cell polarization as shown by formation of leading edge (arrowhead) and trailing edge (arrow) was observed in control but not in FAK-deficient smooth muscle cells. **B**, Representative kymographs of LacZ and Cre-infected cells 0 to 20 minutes following PDGF-BB treatment. Ascending slopes represent protrusion (arrows) and descending slopes represent retraction (arrowheads). **C–E**, Kymographic analysis of lamellipodial dynamics following PDGF treatment. Calculated protrusion rate (**C**), lamellipodial persistence (**D**), and leading edge displacement (**E**) from 8 to 10 cells from 4 independent experiments (mean±SEM; **P*<0.05). **F**, Average 2D cell speed within 4 hours following PDGF treatment. Results are mean± SEM of 30 to 60 cells from 4 independent experiments. **P*<0.05. **G–I**, LacZ or Cre infected *fak*$^{flox/flox}$ cells stained for Rac1 showing dorsal ruffle localization in both control (**G**, top left) and FAK-deficient (**G**, top right) cells at 2.5 minutes following PDGF treatment. At 15 minutes, leading edge localization of Rac1 (arrows) was observed in control cells, whereas FAK-deficient cells showed unpolarized Rac1 localization (arrowheads). **H**, **I**, Quantification of cells exhibiting Rac1-labeled dorsal ruffles 2.5 minutes post-PDGF treatment (**H**) and Rac1-labeled dominant leading edge lamellipodia 15 minutes post-PDGF treatment (**I**). Results are mean±SEM of 150 to 200 cells from 3 independent experiments. ****P*<0.001. **J–L**, Smooth muscle cells treated as above were stained with anticortactin antibody and processed as described in **G–I**. Similar dorsal ruffle but not leading edge recruitment of cortactin was observed in FAK-deficient cells. Results are mean±SEM of 200 to 250 cells from 4 independent experiments. ***P*<0.01. Scale bar=10 μm.

critical role in coordinating and stabilizing the protein complexes that are required for Rac1-dependent leading edge protrusions, and hence, directional SMC motility toward the potent endothelial-derived chemokine, PDGF.

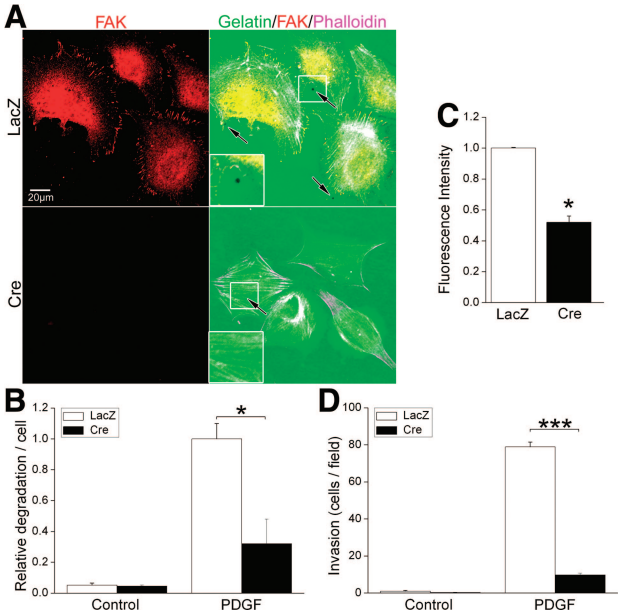

Figure 6. Focal adhesion kinase (FAK) is necessary for smooth muscle cell (SMC)-mediated extracellular matrix degradation. **A**, LacZ- or Cre-infected *fak*$^{flox/flox}$ SMC were plated on Oregon Green 488 gelatin/ fibronectin matrix in serum-free medium and treated with vehicle or platelet-derived growth factor (PDGF)-BB for 90 minutes. Cells were fixed and costained with anti-FAK antibody and phalloidin. **B**, Cells were scored for the presence of degradation puncta (black spots) and data are presented as puncta/cell normalized to values for the LacZ-infected SMC following PDGF treatment. Data represent mean±SEM of at least 200 cells from 3 independent experiments. **P*<0.05. **C**, LacZ- or Cre-infected *fak*$^{flox/flox}$ SMC were plated on DQ-gelatin-coated 96-well plate and treated with PDGF-BB for 90 minutes. Fluorescence intensity was monitored at Ex/Em 495/515 nm. Data represent mean±SEM of 3 independent experiments. **P*<0.05. **D**, GFP and LacZ or Cre coinfected *fak*$^{flox/flox}$ SMC were treated as above were plated on matrigel-coated inserts (10 μg/mL; Bio-Coat) in serum-free media using either PDGF-BB (20 ng/mL) as the chemoattractant. Invading cells were counted by direct fluorescence at 10× magnification. Data represent mean±SEM of 3 independent experiments. ****P*<0.001.

FAK Is Necessary for SMC-Mediated Extracellular Matrix Degradation

SMC are known to have a high basal invasive potential,[25] a function mediated by podosomes, sites of dynamic actin polymerization activity thought to be the topological equivalent of lamellipodia formed during 3D movement. Interestingly, podosomes are initiated at the inner-face of focal adhesion complexes and require cortactin and its associated Arp 2/3 complex for extension into the underlying matrix and for the recruitment and secretion of matrix-degrading proteases.[26,27] To determine whether the chemotactic defect of FAK null SMC in the Boyden chamber assays resulted from impaired directional movement alone or to a combination of impaired motility and invasion, we assessed the capacity of wt and FAK null SMC to degrade extracellular matrix. To this end, we cultured LacZ- or Cre-adenovirus pretreated *fak*$^{flox/flox}$ SMC on fluorescent matrix (Oregon green 488 gelatin/fibronectin mixture) and treated them with or without PDGF for 90 minutes. As shown in Figure 6A, PDGF induced focal areas of matrix degradation (cell associated dark spots) in wt SMC, but this response was significantly

diminished in FAK-deficient SMC. We next evaluated the ability of FAK-containing and FAK null SMC to degrade DQ-gelatin, which fluoresces on degradation allowing quantification of protease activity via fluorescent spectrophotometry. As shown in Figure 6C, significantly lower levels of gelatin degradation occurred in FAK-depleted SMC compared to control SMC, confirming that SMC matrix protease activity is FAK-dependent. In addition, we found that invasion of FAK null SMC into matrigel was significantly impaired in comparison to wt SMC (Figure 6D). These studies indicate that FAK is necessary for both PDGF-stimulated chemotaxis and invasive cell migration; and may explain, at least in part, the selective requirement for FAK in coronary and OFT morphogenesis, as these processes each require SMC to cross tissue/extracellular matrix boundaries.

Discussion

Our studies using 3 independent Cre lines that induce recombination in SM precursor cells revealed a SMC autonomous role for FAK in vascular development. Depletion of FAK in *wnt-1*- and *nkx*2.5-derived cells (ie, FAK^{wnt} and FAK^{nk} embryos, respectively) led to aberrant septation of the great vessels; although depletion of FAK in proepicardial *wt1*-derivatives (ie, FAK^{cSMC} embryos) led to defective coronary vascular formation. Concomitantly, the OFT and coronary vessels were distended by P0, likely due to a block in vessel tone normally imparted by SMC and SMC-elaborated basement membrane components. Phenotypic assessment of each mouse model revealed that the FAK-null SMC were specified but failed to be appropriately recruited to established endothelial tubes. Our studies using FAK-null SMC cultures revealed a selective function for FAK in regulating PDGF-dependent motility but not growth or survival. Specifically, we found that FAK depletion decreased PDGF-stimulated leading edge persistence, cell migration speed, and directional chemotaxis. Given the fact that blockade of PDGF led to defects in recruitment of vascular SMC to the developing vasculature[28,29] and CNC- and epicardial-targeted ablation of PDGF receptor α/β, led to fully penetrant PTA and defective coronary vasculature respectively,[5,7] we speculate that the morphogenesis of these vascular structures involves FAK-dependent SMC motility/chemotaxis induced by endothelial derived PDGFs.

Interestingly, PTA has been associated with a block in SMC differentiation within the truncus of mice harboring null mutations in the BMP receptor, Alk-2 and MRTF-B, as well as those with CNC-targeted expression of a dominant negative Notch variant.[18,30–32] However, similar to our studies, CNC-targeted ablation of $TGF\beta R^2$, PDGFBBR α/β, and Gata 6 each led to fully penetrant PTA without a noticeable reduction in SMC number or SMC maturation.[5,6,33] Interestingly, the interrupted arch phenotype observed in CNC-specific FAK knockout mice recently reported by Vallejo-Illarramemdi et al was attributed to impaired SMC differentiation of a subset of CNC (within the aortic arch), although the authors observed normal differentiation of CNC cells into SMC within the aorticopulmonary septum.[34] Thus, the underlying cause for impaired septation was not identified in the aforementioned manuscript. As we did, this group observed normal migration and growth of CNC in FAK^{wnt} embryos as well as normal differentiation of neural crest cells into SMC within the aorticopulmonary septum. These findings coupled with our mechanistic studies in FAK null SMC suggest that precise regulation of SMC phenotype within the walls of the aortic sac is necessary for subsequent formation of the aorticopulmonary septum. Because PDGF[29] and now FAK have been implicated in the promotion of SMC chemotaxis, it is tempting to speculate that the final septation event may be due to coordinated movement of CNC-derived SMC to the conus. Mis-localization of these cells could therefore lead to impaired coverage of the OFT vessels and impaired force generation within the conus that could impact the dynamic morphogenetic movements during septogenesis.

Because formation of the OFT is extremely complex and involves multiple cell types with interconnected functions, we chose a separate in vivo model (coronary vasculogenesis) to confirm a role for FAK in SMC chemotaxis. The epicardium contains progenitor cells, the major source of SMC that will eventually line the coronary vessels. Elegant lineage tracing studies have shown that SMC precursors are specified in the proepicardium before spreading of this epithelial tissue around the heart.[35–37] In response to signals from the myocardium, these specified cells undergo an epithelial to mesenchyme transition, delaminate from the epicardium, and move into the subepicardial space. Epicardial-derived mesenchymal cells will eventually be induced to migrate into the myocardium and to differentiate into definitive cell types; predominantly the SMC that cover the primary endothelial plexus but also some endothelial and myocardial cells.[20] The endothelial primary plexus originates from the atrial-ventricular groove at the base of the heart and gradually extends to the apex between E11.5-E13.5 in response to prior wave-like secretion of tropic factors from the myocardium (VEGFA and B) and epicardium (sonic hedgehog)[21], an event that is necessary to support midgestational growth. Because the investment of the coronary plexus with mature SMC occurs in a similar (albeit delayed) spatial-temporal pattern, it has been postulated that endothelial-derived factors initiate the recruitment of epicardial-derived SMC. Although the spatial-temporal coupling of epicardial-derived mesenchymal cell differentiation and migration has not been well characterized, the possibility that SMC differentiation may occur before chemotaxis/invasion toward the endothelial plexus is supported by the recent finding that cardiac-restricted depletion of the chemoattractant, thymosin β-4 resulted in robust α-SMA stained cells that aberrantly lined the epicardium (at E14.5 in mouse) but failed to invade the myocardium.[38]

The following findings from our studies support the hypothesis that activation of FAK in SMC by endothelial-derived signals regulates coronary SMC recruitment to (and along) the developing arterioles: (1) Defects were observed in SMC coverage of arterioles in FAK^{cSMC} hearts but not in the formation or function of the endothelial plexus (as assessed by platelet/endothelial cell adhesion molecule staining and appropriate midgestational heart growth); (2) no significant difference was observed in either the rates of proliferation or apoptosis in FAK-null precursor cells, and (3) significantly higher numbers of α-SMA-positive cells were apparent in the

subepicardial space (as opposed to along the endothelial plexus) of the conditional mutants at P0, indicating that FAK was not essential for epicardial-mesenchymal transition but was necessary for movement into the myocardium. These findings coupled with our in vitro evidence that FAK is necessary for chemotaxis/invasion toward the major endothelial-derived chemo-attractant, PDGFBB, indicate that lack of directional motility is likely causal for the SMC investment phenotypes observed in the SMC-selective FAK knock-outs. However, it is formally possible that defects in other parameters not evaluated herein could contribute to the defects observed.

Our mechanistic studies revealed that depletion of FAK markedly impaired PDGF-stimulated formation of a stable leading edge lamellipodium, the hallmark of polarized movement. This defect was accompanied by reduced leading edge recruitment of cortactin, a branch filament stabilizer that is essential for lamellipodial persistence and polarized motility.[39] Moreover, cortactin knock-down was shown to affect lammellipodial activity and directional chemotaxis in exactly the same way as depletion of FAK, suggesting that the functions of these two proteins are interrelated. Interestingly, we found that formation of circular dorsal ruffles in response to PDGF does not require Rac1 activation, cortactin, or FAK. Although the function of these transient dorsal ruffles is not completely understood, there is considerable support for the idea that they are formed as a prelude to leading edge lamella and are important for recruitment and recycling of membrane and actin polymerizing/depolymerizing agents to the presumptive leading edge. Indeed, dorsal ruffles contain many of the same actin regulating components as leading edge lamella including (among others) ARP2/3, WASP1/2, WAVE, dynamin, and cofilin.[40] We surmise that FAK regulates leading edge and not dorsal ruffle formation because FAK is essential for the spatial redistribution of active Rac1 from these complexes to the leading edge.

Because FAK is one of the first proteins recruited to nacient focal complexes, we surmise that FAK initiates recruitment of active Rac1 to these newly formed sites via multifunctional protein complex formation. FAK associates with a number of adapter molecules through well-defined protein interaction sites that could serve as a Rac1 binding platforms. FAK-dependent recruitment of Rac1 could proceed through FAK/(CAS or paxillin)/Crk/DOCK 180 complex formation[41,42] or a paxillin/PIX/COOL complex.[43] Whether FAK-dependent activation of Rac1 at the leading edge is linked to the impaired spatial-temporal leading edge specific redistribution (or phosphorylation) of these or other FAK substrates are important questions for future studies.

There are a few possible explanations for why FAK might be essential for SMC investment of the conotruncus and coronary vasculature, but not for the recruitment of SMC to the distal vasculature (ie, carotid arteries). First, there may be cell type-specific differences in the factors that drive chemotaxis or regional differences in the levels and locale of these so-called motogens. In this regard, it is interesting to note that SMC chemotaxis toward PDGF was exquisitely sensitive to depletion of FAK, although chemotaxis toward serum was only modestly decreased in FAK null SMC (indicating that there are likely FAK-dependent and FAK-independent SMC motogens). The findings that depletion of PDGF receptors from CNC- or proepicardial cells phenocopies the OFT and coronary vessel defects observed in our FAK-depleted models and that PDGFs are expressed in high levels within the conotruncus[5–7] supports the possibility that PDGF and FAK may work in concert to spatially regulate SMC motility in these regions. The second (not mutually exclusive) possibility is that a SMC invasive phenotype (that requires FAK activity) is required for SMC to cross tissue boundaries during both septal formation and coronary vasculogenesis (but not carotid vasculogenesis). Indeed, it may be possible that SMC coverage of carotid vessels occurs by a sheet-like movement/expansion of SMC along the continuously remodeling SMC-lined pharyngeal arches. In support of the possibility that FAK promotes an invasive phenotype, we showed that FAK is necessary for SMC podosome-mediated matrix degradation, and others have recently reported that FAK is essential for invadopodia-mediated matrix degradation in colon cancer cells.[44] Lack of appropriate cortactin recruitment could also be causal for the impaired invasion observed in the FAK null SMC because cortactin has been shown to be necessary for regulating the localized recruitment and secretion of matrix metalloproteinases from lamellipodial-like membrane protrusions.[26] Future studies will be necessary to more fully understand the requirement for SMC invasion versus chemotaxis during the formation of various vascular beds.

In conclusion, our studies indicate that FAK plays a SMC autonomous role in aorticopulmonary septum and coronary vessel formation. Our studies in cultured SMC indicate that FAK activity is critical for SMC chemotaxis/invasion toward PDGF, a potent chemoattractant elaborated from the endothelium. Thus, we surmise that defective PDGF-dependent SMC recruitment leads to improper morphogenesis of FAK null vessels. We found that FAK functions to induce directional SMC motility by regulating the spatial and temporal locale of Rac1-dependent processes, including leading edge recruitment of the actin modifier, cortactin, which is necessary to stabilize lamellipodia and to form productive podosomes. It will be of future interest to evaluate the extent to which FAK may play a role in the pathogenesis of microaneurysms and other vascular diseases associated with defective recruitment of PDGF receptor β positive progenitors.[28]

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Disclosures

None.

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