REGULATION OF PERICYTE BEHAVIOR BY SECRETED PROTEIN ACIDIC AND RICH IN CYSTEINE

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For my family, especially Juliet, Anthony, and my dad.

REGULATION OF PERICYTE BEHAVIOR BY SECRETED PROTEIN ACIDIC AND RICH IN CYSTEINE

by

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ABSTRACT

Pericytes migrate to newly formed vessels where they induce vessel quiescence and promote vessel stability. Secreted Protein Acidic and Rich in Cysteine (SPARC) is a multifunctional matricellular protein believed to be involved in the regulation of vascular cell migration and proliferation during angiogenesis. We previously found that SPARC-deficient mice exhibited abnormal vascular function and decreased pericyte-associated vessels in an orthotopic model of pancreatic ductal

adenocarcinoma (PDAC), suggesting that SPARC regulates pericyte behavior during tumor angiogenesis.

Pericyte expression of SPARC was detected in PDAC lesions of *P48Cre: LSLKras^{G12D}: Ink4A$^{lox/lox}$* mice as well as in normal mouse pancreata by indirect immunofluorescence. Primary mouse pericytes were isolated and the following parameters were characterized: proliferation, migration, the ability to induce bEnd.3 cell cord formation, TGFβ1-induced activity, and TGFβ1 receptor expression.

Here I report that SPARC regulates pericyte migration. I found that SPARC is expressed by pericytes in vivo and confirmed that SPARC-deficient mice have fewer pericyte-associated vessels using a transgenic model of PDAC. Primary pericytes isolated from *SPARC*$^{-/-}$ mice proliferate faster than their *SPARC*$^{+/+}$ counterparts but are less able to induce bEnd.3 cord-formation in vitro. SPARC deficiency also results in defective filopodia and focal adhesion formation and impedes pericyte migration, an effect that is blocked by inhibiting TGFβ. Furthermore, I demonstrate that SPARC interacts with the TGFβ1 accessory receptor endoglin in pericytes. In SPARC-deficient pericytes, endoglin aberrantly associates with focal complexes. SPARC deficiency also induces endoglin-mediated, TGFβ1-induced blockade of pericyte migration, and results in αV integrin-mediated activation of TGFβ1.

These results demonstrate that SPARC controls pericyte migration by regulating endoglin and αV integrin-mediated TGFβ1 activity.

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PRIOR PUBLICATIONS

1.Shanna A. Arnold, **Lee B. Rivera**, Andrew F. Miller, Juliet G. Carbon, Sean P. Dineen, Yang Xie, Diego H. Castrillon, E. Helene Sage, Pauli Puolakkainen, Amy D. Bradshaw and Rolf A. Brekken. (2010) Lack of host SPARC enhances vascular function and tumor spread in an orthotopic murine model of pancreatic carcinoma. *Disease Models and Mechanisms*, 3:57-72. PMCID: PMC2806901

2. Alethia Villasenor, Zhao V. Wang, **Lee B. Rivera**, Ozhan Ocal, Ingrid Wernstedt Asterholm, Philip E. Scherer, Rolf A. Brekken, Ondine Cleaver and Thomas M. Wilkie. (2010) Rgs16 and Rgs8 expression in embryonic endocrine pancreas and mouse models of diabetes. *Disease Models and Mechanism*, (ePub ahead of print) PMID: 20616094

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LIST OF ABBREVIATIONS

^{125}I	^{125} iodine
^{3}H	^{3} hydrogen
ALK1	activin-like kinase 1
ALK5	activin-like kinase 5
Ang-1	angiopoietin 1
α-SMA	alpha smooth muscle actin
BIGH3	TGFβ-induced gene H3
BM-40	basement membrane protein 40
BMP4	bone morphogenic protein 4
BSA	bovine serum albumin
Ca^{+2}	Calcium
cDNA	complementary deoxyribonucleic acid
CNV	choroidal neovascularization
CO_2	carbon dioxide
CTGF	connective tissue growth factor
Cu^{+2}	Copper
DMEM	Dulbeccos modified eagles medium
DNA	deoxyribonucleic acid
DTT	Dithiothreitol
E	embryonic day
ECM	extracellular matrix
EDG-1	endothelial differentiation gene 1
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular regulated kinase
FAK	focal adhesion kinase
Fc	fragment of crystallization
FGF2	fibroblast growth factor 2
FGFR1	fibroblast growth factor receptor 1
GHK	glycine histidine lysine
GIPC	RGS19-interacting protein 1
GTP	guanosine-5'-triphosphate
HB-EGF	heparin-binding epidermal growth factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IgG	immunoglobulin isotype G
ILK	integrin-linked kinase
IP	immunoprecipitation
itgav	αV integrin
itgb6	β6 integrin
kDa	Kilodalton
KO	*SPARC*$^{-/-}$
LAP	latency-associated peptide
LIM	lin-11, isl-1, mec-3 domain
LLC	large latency complex
LPA_2	lysophosphatidic acid2
LSL	lox-stop-lox
LTBP	latency-associated peptide-binding protein
MAPK	mitogen activated protein kinase
MIDAS	metal ion-dependent adhesion site
ml	Milliliter
mm	Millimeter
MMP	matrix metalloprotease
ng	Nanogram
NH_2	Amino
NS	no stimulation
O_2	Oxygen
PAI-1	plasminogen-activated inhibitor 1
PAR-1	protease-activated receptor 1
PAV	pericyte associated vessel
PBS	phosphate-buffered saline
PDAC	pancreatic ductal adenocarcinoma
PDGF	platelet derived growth factor
PDGFAA	platelet derived growth factor-AA
PDGFBB	platelet derived growth factor-BB
PDGFR	platelet derived growth factor receptor
PI3k	phosphoinositide-3 kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIPKIγ	phosphatidylinositol(4) phosphate 5 kinase type I gamma
PKC	protein kinase C
PTP	protein tyrosine phosphatase
PVA	polyvivyl alcohol

qPCR	quatitative polymerase chain reaction
RGD	arginine glycine aspartic acid
RGE	arginine glycine glutamic acid
RNA	ribonucleic acid
ROS	reactive oxygen species
S1P	sphingosine-1-phosphate
SCPP	secretory calcium-binding phosphoprotein
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	standard error margin
SFK	src family kinase
SH2	Src homology domain 2
SHP-1	SH2–containing tyrosine phosphatase-1
shRNA	short hairpin ribonucleic acid
SMAD	homolog of spectrin and mothers against decapentaplegic
SPARC	secreted protein acidic and rich in cysteine
TβRII	transforming growth factor recepter II
TGFβ	transforming growth factor
TGFβ1	transforming growth factor b1
TNF-α	tumor necrosis factor alpha
TRIP6	thyroid hormone receptor interacting protein 6
ug	Microgram
ul	Microliter
um	Micrometer
VASP	vasodilator-stimulated phosphoprotein
VEGF-A	vascular endothelial cell growth factor-A
VEGFR1	vascular endothelial cell growth factor receptor 1
VEGFR2	vascular endothelial cell growth factor receptor 2
VSMC	vascular smooth muscle cells
WCL	whole cell lysate
WT	*SPARC*$^{+/+}$
ZRP-1	zyxin-related protein 1

CHAPTER 1

General information

Problem Statement

Pericytes migrate to newly formed vessels where they induce vessel quiescence and promote vessel stability; however, the molecular mechanisms that regulate pericyte behavior are not fully understood. Modulation of pericyte recruitment to tumor-associated blood vessels is an emerging approach for increasing the efficacy of anti-angiogenic tumor therapy. Currently, such approaches are limited to targeting the PDGF receptor. Further development of anti-pericyte-based anti-angiogenic strategies requires identification of novel factors that could be targeted in conjunction with current approaches. Furthermore, targeting novel factors may prove to be more beneficial to patient outcome compared to existing therapies. Transforming Growth Factor β-1 (TGFβ1) is a pleiotrophic cytokine expressed by endothelial cells and pericytes during angiogenesis. Activation of TGFβ1 occurs upon contact of pericytes with newly formed vessels and inhibits further pericyte migration. The mechanisms that spatially regulate this activation are unclear**.** Secreted Protein Acidic and Rich in Cysteine (SPARC) is a matricellular protein whose expression spatially and temporally overlaps that of TGFβ1 during angiogenesis. Studies in mice have revealed that SPARC is required for appropriate pericyte recruitment during tumor angiogenesis (Puolakkainen et al., 2004). The mechanism behind this effect is unknown; however, these results suggest that modulation of SPARC during tumor growth may be a relevant approach for pericyte-targeted therapy. Recently, SPARC was found to inhibit TGFβ1-mediated fibroblast activation in vivo (Chlenski et al.,

2007). Regulation of TGFβ1 activation by SPARC may therefore represent a novel mechanism through which SPARC can control pericyte behavior. The goal of the work presented in this dissertation was to determine if and how SPARC affects pericyte behavior.

Historical review

Secreted Protein Acidic and Rich in Cysteine (SPARC), also referred to as osteonectin or BM-40, is a 32 kilodalton Ca^{+2} –binding glycoprotein secreted by a variety of cell types. SPARC was originally reported to be a major constituent of non-collagenous fetal calf bone protein extracts (Termine et al., 1981a; Termine et al., 1981b). These initial studies demonstrated SPARC (identified as osteonectin) to be a collagen-binding protein able to facilitate apatite binding and nucleation and to be disproportionately associated with the trabecular matrix rather than the cellular component of bone tissue, effectively characterizing SPARC as a matrix-associated protein and implicating SPARC as an important mediator of bone formation. Indeed, future studies with *SPARC*$^{-/-}$ mice would reveal that lack of SPARC expression results in osteopenia (Mansergh et al., 2007). Subsequently, Sage et al. (Sage et al., 1984) described SPARC as an unidentified albumin-binding protein expressed and secreted by cultured fibroblasts, endothelial cells, vascular smooth muscle cells, and tumor cells. This group purified SPARC secreted by bovine aortic endothelial cells and using a Durrum D-500 amino acid analyzer, found approximately 30% of the amino acid composition of SPARC to consist of acidic residues, and about 3% to consist of cysteine residues. Shortly thereafter, the gene encoding SPARC was cloned from

mouse parietal endoderm cells (Mason et al., 1986). These studies laid the foundation for the study of SPARC and its function. Almost thirty years since its initial discovery, we now know SPARC as a matricellular protein able regulate a myriad of processes including cell migration, cell proliferation, tissue morphogenesis, and tissue repair, and have a more detailed understanding of its molecular and biochemical properties (Brekken and Sage, 2001; Martinek et al., 2007).

SPARC is highly conserved among metazoans (Brekken and Sage, 2001). Interestingly, comparative analyses of several metazoan genomes suggest that SPARC gave rise, through tandem duplication, to the secretory calcium-binding phosphoprotein (SCPP) family of proteins which regulate tissue mineralization (Kawasaki and Weiss, 2006). The human SPARC gene encodes a 303 amino acid protein with a modular structure (Maurer et al., 1995). The full length secreted protein has three distinct domains: domain I is comprised of the 52 NH_2 terminal amino acids, domain II is comprised of residues 53-137, while domain III is comprised of residues 138-286 (Brekken and Sage, 2001; Kaufmann et al., 2004; Maurer et al., 1995). Domain I is an acidic domain with 15 glutamic acid residues. These provide a negative charge and allow this domain to bind Ca^{+2} with low but physiologically significant affinity, ranging from 0.01 to 1.0 mM (Maurer et al., 1995). Domain II is a follistatin-like domain that contains 10 cysteines and a single N-glycosylation site. The follistatin-like region of this domain contains 10 disulfide bonds and forms a motif found in soluble proteins including follistatin, which binds TGFβ superfamily members activin and inhibin, and inhibits binding to their cognate type I and type II

receptors (Hohenester et al., 1997; Thompson et al., 2005; Ying et al., 1987). However, though SPARCs follistatin-like domain is structurally similar to motifs within follistatin, it has not been shown to mediate any interactions between SPARC and TGFβ superfamily members. Domain III is an extracellular Ca^{2+}-binding domain. This domain contains a pair of EF-hands, motifs typically found in Ca^{2+}-binding proteins, as well as an α-helical domain (Maurer et al., 1995). This domain was shown to bind to Ca^{2+} with high affinity (Kd of 0.08 uM) in manner dependent on the interaction between the EF-hand motifs with the α-helical domain (Pottgiesser et al., 1994).

SPARC was originally described as an extracellular matrix (ECM)-associated protein with implications in bone mineralization, and subsequent studies found SPARC to localize to the ECM of developing tissues; therefore much work has been done to characterize the nature of the SPARC-ECM interaction. The ECM provides a physical scaffold for cells within tissues and consists of a variety of molecules, including proteoglycans, growth factors, structural proteins such as fibrillar collagens, collagen IV, fibronectin, and laminin, the proadhesive glycoprotein vitronectin, as well as a group of proteins termed matricellular proteins (Sage and Bornstein, 1991). Matricellular proteins do not provide structural support within or promote adhesion to the ECM; rather, they function to modulate cellular responses to cues from the ECM. SPARC belongs to this group of proteins as it is deadhesive and has neither a cognate receptor nor provides any structural support. Evidence for SPARC's capacity to modulate cell-ECM interactions first came from studies demonstrating that addition of

purified SPARC to cells in vitro reduced cell spreading and induced cell rounding, suggesting that SPARC controlled the interaction between the cell and its substratum (Sage et al., 1989). Further studies revealed that SPARC added to the media of a variety of cell types altered their interaction with their substrates. This deadhesive activity has been mapped to SPARC's extracellular Ca^{2+} -binding domain (Brekken and Sage, 2001). SPARC's ability to function as a matricellular protein and thus control cellular responses to the ECM may rely in part on its ability to interact with the ECM. To date, SPARC has been shown to bind several components of the ECM, including vitronectin, laminin-1, collagens I-V, and collagen VIII (Bradshaw, 2009; Brekken et al., 2003).

SPARC can also control remodeling and formation of the ECM. Tremble et al. (Tremble et al., 1993) demonstrated that incubation of rabbit synovial fibroblasts with recombinant SPARC resulted in increased expression and secretion of collagen-degrading (types I, II, and III) matrix-metalloprotease 1 (MMP1) and the more promiscuous matrix-metalloprotease 3 (MMP3), which degrades collagens III, IV, IX, X, fibronectin, and laminin. These enzymes are expressed and activated during remodeling of the ECM (Nagase et al., 1999). Studies of *SPARC*$^{-/-}$ mice have revealed that SPARC regulates collagen processing and maturation (Bradshaw, 2009). Collagen fibrils were first shown to be highly irregular in the skin of *SPARC*$^{-/-}$ mice, with a large proportion of these fibers having abnormally small diameters (Bradshaw et al., 2003). Later, SPARC was found to inhibit fibrillogenesis of bovine type I collagen, suggesting that SPARC may directly regulate fibril assembly in vivo

(Giudici et al., 2008). SPARC can also regulate the processing of secreted collagen. Fibroblasts isolated from *SPARC*$^{-/-}$ mouse dermis secreted less collagen into the culture media; rather, it was preferentially retained in the cell layer (Rentz et al., 2007). Furthermore, *SPARC*$^{-/-}$ fibroblasts secreted collagen faster than *SPARC*$^{+/+}$ fibroblasts, but incorporation into detergent insoluble protein, or mature collagen ECM, was less efficient. The study also found that *SPARC*$^{-/-}$ fibroblast-conditioned media contained more procollagen processing products than *SPARC*$^{+/+}$ fibroblast-conditioned media, suggesting that SPARC regulates collagen processing, which allows for proper ECM synthesis and maturation.

In addition to its function as a regulator of ECM formation, SPARC has been shown to influence a variety growth factor signaling pathways by directly interacting with the soluble growth factor or affecting activity of the ligand-bound growth factor receptor. The first such report identified and characterized the interaction of platelet-derived growth factor (PDGF) with SPARC (Raines et al., 1992). PDGF is a mitogenic and chemotactic growth factor typically secreted by mesenchymal cells and participates in development and disease. PDGF is secreted as a dimer of two disulfide-linked glycoproteins. The secreted growth factor can be either a homodimer of two alpha (PDGF-AA) or beta (PDGF-BB) chains, or a heterodimer (PDGF-AB), having both an alpha and a beta chain. There are two cognate receptor tyrosine kinases which specifically interact with PDGF: PDGF-receptor alpha (PDGFRα) and PDGF-receptor beta (PDGFRβ). PDGFRα binds to all three PDGF dimers, while PDGFRβ preferentially recognizes PDGF-BB and PDGF-AB, having a lower affinity for the

AA dimer (Tallquist and Kazlauskas, 2004). Upon ligation of PDGF, the receptors dimerize resulting in either an αα, ββ, or αβ receptor complex. SPARC can regulate PDGF receptor activity by controlling ligand access via direct ligand interaction. This interaction is specific for PDFG-AB and PDGF-BB. SPARC, or a synthetic peptide with anti-spreading properties corresponding to SPARC residues 5-23 (peptide 1.1), co-immunoprecipitated ^{125}I-labeled PDGF-AB and PDGF-BB, but not PDGF-AA (Raines et al., 1992). This group went on to show that SPARC blocked the binding of PDGF-AB and PDGF-BB to human fibroblasts, while having no effect of the binding of PDGF-AA. Another study found that SPARC was able to block PDGFB-induced proliferation of human arterial smooth muscle cells, suggesting that SPARCs inhibitory activity is physiologically relevant (Motamed et al., 2002). SPARC was later shown to interact with vascular endothelial growth factor-A (VEGF-A) (Kupprion et al., 1998). VEGF-A is a member of a subfamily of the PDGF family of growth factors. There are three known receptor tyrosine kinases which bind to members of this subfamily, but only VEGF-receptor 1 (VEGFR1) and VEGF-receptor 2 (VEGFR2) recognize VEGF-A (Sullivan et al.). ^{125}I-labeled SPARC was demonstrated to bind immobilized VEGF-A; this interaction was inhibited by unlabeled SPARC (Kupprion et al., 1998). The group went on to show that a peptide corresponding to SPARC residues 254-273 (peptide 4.2) blocked binding of ^{125}I-labeled VEGF-A to human microvascular endothelial cells, and that SPARC specifically blocked VEGF-A-induced VEGFR1 phosphorylation while having no effect on VEGFR2 phosphorylation, suggesting that SPARC specifically blocks the

interaction between VEGF-A and VEGFR1. Molecular docking simulations have predicted that the extracellular Ca^{2+}-binding domain of SPARC interacts with the VEGFR1-binding site of VEGF-A, supporting this theory (Chandrasekaran et al., 2007). SPARC has also been shown to interact with the fibroblast growth factor 2 (FGF2)/ fibroblast growth factor receptor 1 (FGFR1) signaling axis, though not through a direct interaction with the soluble growth factor (Hasselaar and Sage, 1992; Motamed et al., 2003). FGF2 is a member of the fibroblast growth factor (FGF) family of growth factors. FGF2 binds and signals through the fibroblast growth factor receptor (FGFR) family of receptor tyrosine kinases, and formation of a ternary complex FGF-bound FGFR with heparan sulfate proteoglycans is required for many FGF-induced responses (Quarto and Amalric, 1994). SPARC was shown to inhibit FGF2-induced migration of bovine endothelial cells without inhibiting the interaction of ^{125}I-labeled FGF2 with the cells (Hasselaar and Sage, 1992). Also, biotinylated SPARC, even at a three-fold molar excess, was unable to interfere with the interaction between FGF2 and recombinant human FGFR1-Fc chimeric protein (Motamed et al., 2003). The group went on to show that SPARC was able to inhibit FGF2-induced phosphorylation of FGFR1, MAPK activation, and DNA synthesis and did not require heparan sulfate proteoglycans. To further confirm that SPARC was also able to inhibit FGF2/FGFR1 signaling, the group used the murine myoblast MM14 cell line, which requires FGF2/FGFR1 signaling to maintain an undifferentiated state (Templeton and Hauschka, 1992). Addition of SPARC or peptide 1.1 blocked the effect of FGF2 and induced MM14 terminal differentiation.

As SPARC is matricellular protein, able to modulate cell/ECM interactions and influence cell responses to growth factors, it seems especially attuned to physiological processes involving changes in ECM and cell mobilization. Indeed, SPARC has been shown to play a role in wound healing, tumor progression, bone formation, and angiogenesis (Clark and Sage, 2008; Jendraschak and Sage, 1996; Martinek et al., 2007).

Implications that SPARC is a regulator of angiogenesis

Review of angiogenesis

Angiogenesis is a critical function of the blood vascular system; it entails a complex cascade of molecular events that ultimately result in the formation of new microvessels from an existing vascular bed. This process has been demonstrated to be required not only for normal development but also disease progression. The microvasculature compartment consists of highly permeable capillaries, which are physically embedded within all tissues and serve to provide tissues with O_2 and nutrients and pick up CO_2 for transport to the lungs. Oxygenated blood reaches the capillary bed by way of arteries. Arteries transport blood from the lungs and heart to the tissues. They are continuous with arterioles and transition into capillaries, which are the principle site of O_2, nutrient, and waste product exchange within the body. Capillaries then transition into post-capillary venules which are continuous with veins that transport nutrient and O_2-depleted blood back to the lungs.

Angiogenesis first occurs after vasculogenesis (the formation of the primary vascular plexus from hemangioblasts). Vasculogenesis is an early event in embryonic development and is required for the development of subsequent tissues. Practically, the O_2 diffusion limit is approximately 200 μm, so tissues must grow in parallel with the vasculature in order to maintain viable O_2 levels (Klagsbrun and Eichmann, 2005). Indeed, many mutations that interfere with this process result in embryonic lethality (Murakami et al., 2008). Vasculogenesis begins with the formation of vascular progenitor cells called hemangioblasts which arise from the mesoderm. These cells are induced in response to FGF2 and bone morphogenic protein 4 (BMP4) signaling, express VEGFR2, and are able to give rise to both blood and endothelial cells (Oh et al., 1997). The hemangioblasts differentiate into either endothelial cell-lined blood islands which, in response to VEGF-A, undergo fusion to form a primary capillary plexus, or into the dorsal aorta and cardinal vein. These vessels fuse and undergo remodeling, resulting in a functional vascular network. Secretion of PDGF by vascular endothelial cells recruits mesenchymal cells that become either pericytes, which line the capillaries, or vascular smooth muscles cells (VSMCs), which line the larger vessels (Kutcher and Herman, 2009). Association of these cells with the nascent vasculature results in stabilization and quiescence of the vessel network. This includes an intact microvascular compartment that is able to supply the surrounding tissue with O_2 and nutrients. Angiogenesis also occurs post developmentally during times where tissue mass has grown past the O_2 diffusion limit set by the resident capillaries, such as during tumor development. Many of the pathways involved with developmental

angiogenesis have also been shown to be critical regulators of post developmental angiogenesis. Briefly, post developmental angiogenesis involves VEGF-A-mediated mobilization of capillary endothelial cells. These migrate and proliferate to form new endothelial tubes which then secrete factors such as PDGF which induce pericyte or VSMC migration and recruitment, resulting in vessel stabilization.

The quiescent adult microvasculature consists of two cells types: the endothelial cell and the pericyte (Fig. 1.1). Endothelial cells make up the tubular structure of the capillary network. As with all other vessels of the blood vascular system, these are the cells that provide the vessel lumen. Capillary lumen diameter varies depending on the tissue type, but typically lies between 5 and 10 um (Nelson, 1999). The endothelial cells are anchored to the surrounding basement membrane and are in contact with each other via tight junction interactions; these junctions allow molecules less than 75 kDa to escape the vessel and diffuse into the surrounding tissue (Cheresh and Stupack, 2008). Unlike the constituent endothelial cells, pericytes do not form a continuous sheath or contribute to the tubular capillary network. Instead, they are found as single cells, distributed at intervals along the endothelial-capillary tube. Pericytes reside at the interface between the extracellular environment and the capillary tube and are physically embedded in the capillary basement membrane (Kutcher and Herman, 2009). These cells protrude plasma membrane extensions over the endothelial tube and are believed to provide survival signals to the underlying endothelial layer and are required for vessel stabilization. Therefore, the result of an

angiogenic stimulus such as VEGF-A depends on an endothelial cell response and is also dictated by the activity of the resident pericytes.

Early implications of SPARC as a regulation of angiogenesis

Early on, studies of SPARC and its function revealed that it may participate in angiogenesis. SPARC was initially isolated in vitro from cultured endothelial cells, and expression was later shown to be induced upon induction of various stresses (Sage, 1986; Sage et al., 1984; Sage et al., 1986). Paradoxically, SPARC expression was initially found in proliferating endothelial cells, while addition of purified SPARC to cultured endothelial cells was shown to decrease proliferation. Addition of purified SPARC to bovine aortic endothelial cells was able to inhibit ^{3}H-thymidine incorporation and the onset of S-phase in a dose dependent manner (Funk and Sage, 1991). This anti-proliferative effect was functionally mapped to SPARC residues 54-73 and 184-203 (peptides 2.1 and 3.4, respectively). Exogenous SPARC was subsequently shown to have differential effects on endothelial cells and fibroblasts, suggesting that SPARCs effects on proliferation are not direct (Funk 1993). Addition of peptide 2.1 to human foreskin fibroblasts or bovine ligament fibroblasts stimulated ^{3}H-thymidine incorporation but inhibited ^{3}H-thymidine incorporation in transformed bovine aortic endothelial cells, bovine capillary endothelial cells, and human umbilical vein endothelial cells (Funk and Sage, 1993). Another peptide corresponding to SPARC residues 113-130 (peptide 2.3) was able to induce ^{3}H-thymidine incorporation in both endothelial cells and fibroblasts (Funk and Sage,

1993). Interestingly, this peptide contains a Cu^{2+} binding sequence, GHK. GHK peptides act as serum mitogens and can induce angiogenesis in vivo (Pickart and Lovejoy, 1987). In vitro studies demonstrated that peptide 2.3 was able to induce bovine aortic endothelial cell tube formation and that cleavage of SPARC by the angiogenic protease plasmin resulted in the release of peptides containing the GHK sequence (Lane et al., 1994; Thompson et al., 1996).

Evidence from animal studies

Early animal studies have also implicated SPARC as being a regulator of angiogenesis. Immunohistochemical detection and in situ hybridization studies have revealed that SPARC is expressed in embryonic brain capillaries, dermal wounds capillaries, and in newly formed vessels of the allantoic membrane (Iruela-Arispe et al., 1995; Lane et al., 1994). Studies in *SPARC*$^{-/-}$ mice have demonstrated that SPARC is required for angiogenic responses, though whether it serves as a promoter or inhibitor of the response is dependent on the model being used. One such model is the polyvinyl alcohol (PVA) sponge. PVA sponges illicit an angiogenic response similar to that observed during wound healing when implanted subcutaneously (Andrade et al., 1997). Sponges implanted into *SPARC*$^{-/-}$ mice exhibited increased VEGFR2-immunoreative microvessels compared to sponges implanted into *SPARC*$^{+/+}$ mice 12 and 20 days after implantation (Bradshaw et al., 2001). This relative increase in microvessel formation was accompanied by a dramatic increase in red blood cell content. Interestingly, this enhanced angiogenic response was shown to dampen as age

of the recipient animal increased (Reed et al., 2005). Polydimethylsiloxane disks (silicone disks) have also been implanted into *SPARC*$^{-/-}$ mice, however the results imply an inhibitory role for SPARC. Silicone disks, like PVA sponges, illicit an angiogenic response (Kyriakides et al., 1999) Implantation into *SPARC*$^{-/-}$ mice resulted in decreased vascular area in the foreign body response capsule surrounding the disk compared to *SPARC*$^{+/+}$ mice 4 weeks after implantation (Puolakkainen et al., 2003). The apparent disagreement between results is likely due in part to the angiogenic response being subject to the nature synthetic materials used for each model. The PVA sponge model does eventually illicit a foreign body response; however, during the first 3 weeks, the implants induce marked fibrotic and vascular invasion due to the inherent porosity of the material (Kyriakides and Bornstein, 2003). The porosity may allow the invading cells opportunity to express growth factors and provisional matrix proteins such as fibronectin and is more conducive to the diffusion of growth factors (Kyriakides et al., 2001). As SPARC has been shown to enhance fibronectin-induced stress fiber formation and block angiogenic growth factors in vitro, the microenvironment within the PVA sponge during the first 3 weeks after implantation may be subject to SPARC's control of these processes, which would be consistent with the results of (Bradshaw et al., 2001; Reed et al., 2005). In contrast, silicone disks lack such porosity and induce fibrotic capsule formation soon after implantation (Qiu et al., 1998). These capsules are rich in collagen, exhibit very low molecular diffusion rates, and seem to function as barriers, blocking the disks from the surrounding tissue (Sharkawy et al., 1997; Vistnes et al., 1978). These factors may

create a microenvironment that is subject to other functions of SPARC that result in decreased vascular area. Another model used to study the effect of SPARC on angiogenesis is the subcutaneous or orthotopic injection of tumor cells. Implanted tumor cells will only grow to only 1 or 2 mm^3 before they stimulate angiogenesis (Folkman, 1974).This model is different than the PVA sponge and silicone disk models in that both the sponge and disk are completely acellular, synthetic implants, free of any biologically active factors. However, the tumor model is similar to the PVA sponge model in at least 2 respects. First, both of the model-induced responses are initially very similar to what is observed in wound healing in that there is extensive fibrotic and vascular invasion (Orimo and Weinberg, 2006). Second, unlike wound healing, which is characterized by an early inflammatory state (in which extensive remodeling occurs) that resolves as the tissue heals, both the PVA sponge model and the tumor implant model exist in a state of chronic inflammation. Despite these similarities, studies of tumor implant-induced angiogenesis have yielded results more in line with those seen with the silicone disk model. Brekken et al. (Brekken et al., 2003) first showed that LLC cells (Lewis Lung carcinoma) grown subcutaneously in *SPARC*$^{-/-}$ mice exhibited reduced vascular area compared to those grown in *SPARC*$^{+/+}$ mice. Puolakkainen et al. (Puolakkainen et al., 2004) then demonstrated that Pan02 cells (mouse pancreatic carcinoma) grown subcutaneously into *SPARC*$^{-/-}$ mice exhibited decreased pericyte-associated vessels (using smooth muscle actin as pericyte marker) compared to those grown in *SPARC*$^{+/+}$. These results suggest that SPARC positively regulates tumor angiogenesis. In support of this, we found that Pan02

tumors grown in the pancreata of *SPARC*$^{-/-}$ mice exhibited a decrease in both vessel number and vessel maturity compared to those grown in *SPARC*$^{+/+}$ mice (Arnold et al.). Though studies with synthetic and cellular implants have yielded results that are at first glance contradictory, they all implicate SPARC as a regulator of the angiogenic process and highlight the complexity of SPARC function in vivo.

SPARC and the angiogenic cascade

Angiogenesis is critical to diseases such as cancer therefore understanding the underlying events that drive it are of immediate importance. The molecular pathways responsible for angiogenesis have therefore been the subject of much attention for the past several decades (Folkman et al., 1971). There is a large consensus among the scientific and medical communities that the angiogenic process occurs in a stepwise manner (Bryan and D'Amore, 2007; Chantrain et al., 2006; Folkman and D'Amore, 1996). Briefly, an angiogenic stimulus, such as VEGF-A, is secreted by cells under certain conditions such as hypoxia. This stimulates endothelial cell proliferation and production of MMPs which degrade the surrounding basement membrane, detaching the endothelial cell layer from adjoining pericytes and liberating ECM-sequestered growth factors. The stimulated endothelial cells then migrate from the existing capillary and coalesce to form a tube-like, lumen-containing structure. Endothelial cells also actively secrete factors that induce proliferation and migration of the resident pericyte population. This results in pericyte recruitment to the newly formed endothelial tube. Pericyte/endothelial cell interactions then promote stabilization and

quiescence of the nascent vessel. The study of SPARC has yielded findings implicating involvement of SPARC with each of these processes (Table 1).

Angiogenic molecules regulated by SPARC

Molecule	Role in angiogenesis	Effect of SPARC	references
VEGF-A	Stimulates endothelial cell activation and initiates angiogenesis through VEGFR2.	SPARC directly interacts with VEGF-A and prevents it from inducing VEGFR1 activity in cultured endothelial cells and in vivo. VEGF-A is predicted to interact with SPARC at it's VEGFR1-binding site.	Kupprion 1998, Chandrasekaran 2007, Nozaki 2006.
FGF2	Stimulates endothelial cell activation and initiates angiogenesis through FGFR1.	SPARC inhibits the FGF2-induced activation of endothelial cells in vitro.	Motamed 2003.
PDGFB	Stimulates pericyte proliferation and migation through PDGFRβ.	SPARC directly interacts with PDGFBB and PDGFAB and prevents PDGF-induced responses in fibroblasts and mural cells in vitro.	Raines 1992, Motamed 2002.
TGFβ1	Can stimulate endothelial cell activation through ALK1 and inhibit activation through ALK5. Inhibits pericyte migration and induces their differentiation through ALK5.	TGFβ1 induces expression of SPARC in a variety of cell types including fibroblasts. SPARC also regulates expression of TGFβ1. Activation of TGFβ1 can be induced or inhibited by SPARC.	Wrana 1991, Ford 1993, Reed 1993, Schiemann 2003, Francki 2004, Chlenski 2007.
MMPs 1, 2, 3,9, 14	Degradation of the surrounding basement membrane and ECM. Liberatation of sequestered angiogenic growth factors	SPARC induces expression and activity of MMPs 1, 2, 3, 9, and 14 in fibroblasts and various tumor cell lines.	Tremble 1993, Gile 1998, Fujita 2002, McClung 2007.
α6 integrin	Heterodimerizes with β1 and β4 integrins to form laminin receptors. Effects of these integrins on angiogenesis vary. Their role is inconclusive.	SPARC decreases expression and activity of α6β1 in lens epithelial cells and α6 integrins in preadipocytes in vitro.	Weaver 2006, Nie 2008.
β1 integrin	Forms laminin receptors with α3 and α6, forms fibronectin receptors with α4 and α5, forms collagen receptors with α1 and α2 in endothelial cells and pericytes. Mediates pericyte recruitment and endothelial survival.	SPARC interacts with β1 integrin and induces ILK activity in lens epithelial cells	Weaver 2008.
αvβ3 and αvβ5 integrins	Vitronectin receptors (also bind fibrinogen and fibronectin). Induce endothelial cell survival in response to ligand interaction, and induce cell death in the absence of ligand.	SPARC can increase αvβ3 and αvβ5-mediated migration of prostate tumor cells and dental pulp cells in vitro. SPARC blocks αvβ3 and αvβ5-mediated attachment and surface expression in several ovarian cancer cell lines.	De 2006, Pavasant 2007, Said 2006, Said 2007.

Table 1. **Angiogenic molecules regulated by SPARC.** SPARC regulates a variety of molecules with important roles in angiogenesis. See text for details.

Vascular endothelial growth factor-A and Fibroblast growth factor 2

As the adult vasculature is largely quiescent, the production of angiogenic factors is tightly regulated and is stimulated only under certain conditions, such as hypoxia (Giordano 2001). Indeed, expression of the prototypical angiogenic factor VEGF-A was found to be elevated during hypoxia almost 20 years ago (Shweiki et al., 1992). VEGF-A drives angiogenesis by binding to and activating VEGFR2, which results in receptor autophosphorylation. Active VEGFR2 stimulates phosphoinositide-3 kinase (PI3K), which promotes vascular permeability as well as endothelial cell survival (Jiang et al., 2009) and the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) pathways. Downstream p38 effectors function to induce endothelial cell migration, while ERK pathway effectors stimulate progression through the cell cycle (Rousseau et al., 1997; Schultz, 2003). Endothelial cells also express VEGFR1 which has a higher affinity for VEGF-A and antagonizes VEGF-A/VEGFR2 stimulated processes, however, the signals transduced through this interaction are not clear (Hiratsuka et al., 2005). It is widely held that one of the primary functions of the VEGF-A/VEGFR1 interaction is to sequester VEGF from VEGFR2, thereby preventing activation of the pathways that promote endothelial survival and induce migration and proliferation. However, some studies indicate that VEGF-A-bound VEGFR1 may actively regulate specific intracellular signaling pathways stimulated by VEGF-A-bound VEGFR2 (Zeng et al., 2001). As previously discussed, SPARC binds to VEGF-A, an interaction presumably mediated by a region within SPARC residues 254-273. This results in decreased phosphorylation of VEGFR1 while having no effect on the phosphorylation of

VEGFR2 (Kupprion et al., 1998). Nozaki et al. (Nozaki et al., 2006) demonstrated that blockade of the VEGF-A/VEGFR1 interaction by SPARC allows for VEGF-A/VEGFR2 signaling to drive angiogenesis. Using a model of choroidal neovascularization (CNV) in which a laser is used to induce an angiogenic response in the eye, this group demonstrated that VEGF-A inhibited angiogenesis if it was injected into the animal after injury, but induced angiogenesis if added before injury administration. The anti-angiogenic effect of VEGF-A post injury was reversed if the animals were treated with a VEGFR1-blocking antibody, suggesting that VEGFR1 mediated the inhibition of VEGF-A-induced angiogenesis. The group found that SPARC was constitutively expressed in the region of the eye assayed but that expression decreased over time after CNV was induced, suggesting that SPARC may block the antagonistic effect of VEGF-A/VEGFR1 at the time of injury, thereby allowing VEGF-A to activate VEGFR2 signaling to drive angiogenesis. In support of this, VEGF-A was unable to stimulate CNV when administered before injury in *SPARC*$^{-/-}$ mice. That SPARC levels decreased over time after injury was consistent with their data showing that VEGF-A was able to block CNV if administered post injury. Indeed, injection of recombinant SPARC blocked the inhibitory of effect of VEGF-A on CNV post injury. Interestingly, VEGF-A-mediated inhibition of CNV was associated with an increase in the association of protein tyrosine phosphatase (PTP) Src homology domain 2–containing (SH2-containing) tyrosine phosphatase-1 (SHP-1) with VEGFR2, and a concomitant decrease in VEGFR2 tyrosine phosphorylation. Also, the antagonistic effect of VEGF-A post injury was dependent

on phosphatase activity. These results suggest that SPARC may function to block VEGFR1-mediated recruitment of SHP-1 to VEGFR2, thereby allowing phosphorylated VEGFR2 to induce an angiogenic response.

Another secreted factor that has the capacity to induce angiogenesis is FGF2. FGF2 was the first angiogenic factor discovered, originally purified and characterized by virtue of its heparan-binding ability (Shing et al., 1984). Signaling of FGF2 through the FGFR1 receptor has been shown to be required for development of the vasculature and to be important during tumor-induced angiogenesis (Lee and Kay, 2006; Nissen et al., 2003). Binding of FGF2 by FGFR1 induces receptor dimerization and autophosphorylation. The activated receptor then induces many of the same intracellular signaling cascades as activated VEGFR2 which promote endothelial cell survival and induce proliferation and migration (Cross and Claesson-Welsh, 2001). Experiments with human microvascular cells have shown that SPARC can inhibit the effect of FGF2-induced proliferation (Motamed et al., 2003). Treatment of these cells with SPARC blocked FGF2-induced DNA synthesis, MAPK phosphorylation, and FGFR1 phosphorylation. Interestingly, this inhibitory activity of SPARC was mapped to a region within SPARC residues 254-273, the same region that binds to VEGF-A and inhibits interaction of VEGF-A with VEGFR1, suggesting that SPARC's effects on FGF2 signaling may be linked to effects on VEGF-A receptor signaling. In agreement with this is the inability of the authors of this study to demonstrate an interaction between either SPARC and FGF2, or SPARC and FGFR1, and the fact that SPARC did not interfere with binding of FGF2 to FGFR1 (Motamed et al., 2003).

Also, the cells used in these experiments likely express some level of endogenous VEGF-A as autocrine VEGF-A signaling is required for endothelial cell survival (Lee S. 2007). A recent report demonstrated that an autocrine VEGF-A/VEGFR1 signaling axis was required for EGF/EGFR-induced proliferation of squamous cell carcinoma cells (Lichtenberger et al.). The results from this study open up the possibility that autocrine VEGF-A/VEGFR1 signaling may be required for the proliferative effects of other growth factors such as FGF2, where blockade of VEGF-A from interacting with VEGFR1 by SPARC may decrease FGF2-induced signal transduction through FGFR1.

Matrix Metalloproteases

Stimulation of quiescent endothelial cells by VEGF-A or FGF2 is followed by their rapid mobilization into the surrounding tissue. This requires degradation of the surrounding capillary basement membrane as well as of the surrounding ECM. Degradation of these compartments is mediated in part by MMPs. MMPs are extracellular proteases that generally exist as proenzmyes whose expression is induced early in angiogenesis (Stetler-Stevenson, 2008). All MMPs contain a prodomain, catalytic domain, hinge domain, and a hemopexin domain. Activation of the proenzyme involves cleavage of the hemopexin domain which can be mediated by other proteases, low pH, or by chemical modification (De et al., 1999; Suzuki and Katoh, 1990). Once activated, MMPs actively degrade structural components of the ECM and basement membrane. This not only removes a constraint of cellular

migration but releases ECM-sequestered growth factors as well. SPARC was found to regulate rabbit synovial fibroblast expression of collagen degrading MMPs MMP1, MMP3, and MMP9, all of which regulate angiogenesis, almost two decades ago (Tremble et al., 1993). In addition to degrading the surrounding matrix, MMP1 can cleave protease-activated receptor 1 (PAR-1) expressed on microvessel endothelial cell surfaces, which results in PAR-1 mediated activation of MAPK signaling and tube formation (Blackburn and Brinckerhoff, 2008). MMP1 can also liberate connective tissue growth factor (CTGF)-bound VEGF-A in the ECM, making it available to bind VEGFR2 on endothelial cells (Raffetto and Khalil, 2008). MMP3 can liberate sequestered VEGF-A, and along with MMP9, can activate TGFβ1, which stimulates proliferation and migration of endothelial cells through activin-like kinase 1 (ALK1) and induces pericyte quiescence via activin-like kinase 5 (ALK5) (Lebrin et al., 2004). Interestingly MMP3 activates TGFβ1 by degrading decorin, a collagen-binding protein that sequesters active TGFβ1 to the ECM, while MMP9 activates TGFβ1 by releasing the latency-associated peptide (Annes et al., 2003). SPARC can also activate MMP2 and regulate the expression of the membrane-bound MMP14 (Gilles et al., 1998). Addition of recombinant SPARC to human breast cancer cell lines MDA-MB-231 and BT549 induced gelatinase activity of MMP2 as detected by gelatin zymography (Gilles et al., 1998). Like MMPs 3 and 9, MMP2 can activate latent TGFβ1 as well as release decorin-bound active TGFβ1 (Imai et al., 1997). This group also demonstrated that recombinant SPARC was able to induce expression of MMP14 (Gilles et al., 1998). MMP14 is critical to both endothelial cell and pericyte

migration and can activate the proforms of many other MMPs (Stawowy et al., 2004). Though these studies were not done using vascular cells, they demonstrate that SPARC can control the expression of many MMPs required for angiogenesis. As SPARC expression is markedly induced in endothelial cells during angiogenesis, it is logical to hypothesize that SPARC may function to regulate the expression or activity of MMPs during angiogenesis.

Platelet derived growth factor

Nascent endothelial tubes require the association of pericytes for their stabilization. Pericyte recruitment to nascent endothelial tubes is mediated by several signaling cascades including sphingosine-1 phosphate (S1P)/ endothelial differentiation gene-1(EDG-1), heparin-binding epidermal growth factor (HB-EGF)/ EGFR, and PDGFB/ PDGFRβ. Pericytes proliferate in response to these factors and migrate to the nascent endothelial tube where they induce vessel quiescence. The PDGFB/PDGFR pathway was the first of these to be shown to be required for angiogenesis. Lack of PDGFB expression resulted in the failure of vessels to recruit pericytes resulting in microaneurisms and embryonic lethality (Lindahl et al., 1997). Binding of PDGFBB or PDGFAB to PDGFRβ induces activation Ras/Rho/Rac as well as protein kinase C (PKC), all of which act in concert to induce pericyte migration. Ras also activates FAK and ERK, which induce pericyte proliferation. SPARC binds to PDGF-BB and PDGF-AB and prevents binding to their cognate receptors and PDGF-induced proliferation of fibroblasts and mural cells (Motamed et

al., 2002; Raines et al., 1992). This suggests that SPARC can also function to control the effect of PDGF-B on pericytes in vivo. As SPARC is a substrate for MMP3, one possibility is that the inhibitory effect of SPARC on PDGF-B-induced pericyte responses may depend on the balance of SPARC and MMP3 activity. As MMP3 secretion and activation increases during angiogenesis, MMP3-mediated SPARC degradation may provide mechanism with which the angiogenic microenvironment can use to temporally regulate PDGF-B-induced pericyte responses, where early on, PDGF-B activity is blocked by SPARC, and later on, perhaps after tube formation has occurred, SPARC is degraded and unable to block PDGF-B-induced pericyte behavior.

Transforming growth factor-β1

Upon association of pericytes with endothelial cells, TGFβ1 activation induces pericyte maturation and blood vessel stabilization. TGFβ1 belongs to the TGFβ superfamily of cytokines which includes TGFβ2 and 3, bone morphogenic proteins (BMPs), activins, and inhibins. Genetic ablation of TGFβ1 results in abnormal vessel formation and embryonic lethality in mice (Dickson et al., 1995a; Dickson et al., 1995b). Translated TGFβ1 contains a latency associated peptide (LAP) which associates with a latent TGFβ- binding protein (LTBP). LTBP functions to dock the latent TGFβ1 protein complex to the ECM, before being secreted out of the cell (Saharinen et al., 1999). Binding of secreted TGFβ1 to its cognate receptors is blocked by LAP, therefore activation requires LAP cleavage or the pulling of LAP to

expose the receptor binding region of TGFβ1 (Lyons et al., 1988; Sheppard, 2001). There are 3 type of TGFβ receptors which sequentially interact with TGFβ1. Active TGFβ1 first binds to a homo-dimer of the type II TGFβ receptor (TβRII). TβRII is a serine/threonine kinase and TGFβ1 ligation induces autophosphorylation of the receptor dimer and promotes the association with a dimer of a type-I TGFβ receptor (activin-like kinase or ALK), another serine/threonine kinase. Similar to TGFβ, ALK receptors are required for normal development of the vasculature (Larson et al., 2001; Oh et al., 2000; Urness et al., 2000). Animals lacking ALK receptor expression die during embryonic development and exhibit unstable vessels with significantly reduced pericyte coverage, suggesting that ALK signaling is a critical mediator of pericyte recruitment. The ALK dimer binds to both TGFβ1 and to TβRII and is responsible for phosphorylating specific R-SMAD transcription factors, which are associated with the intracellular face of the cell membrane (Massague, 1998). R-SMADs mediate many of the downstream events following TGFβ1/receptor activation including expression of α-smooth muscle actin (α–SMA) and extracellular matrix proteins (Lonn et al., 2009). Phosphorylated R-SMADs require association with the Co-SMAD, SMAD4, to accumulate in the nucleus where they interact with chromatin to regulate gene transcription. SMAD4 is required for angiogenesis as endothelial cell-specific knockout results in defects in vascular remodeling during development, resulting in embryonic lethality (Lan et al., 2007). The activated receptor complex of TGFβ1/TβRII/ALK can then associate with an accessory receptor such as betaglycan or endoglin. Though the function of these interactions in not well understood,

accessory receptor expression is critical to development of the vasculature, as loss of endoglin expression during development prevents normal blood vessel formation and results in embryonic lethality (Li et al., 1999). One possibility is that the accessory receptors modulate intracellular function of the activated receptors. For example, the association of endoglin with the receptor complex results in altered phosphorylation states of the receptors and SMAD proteins (Guerrero-Esteo et al., 2002). As the intracellular domain of endoglin does not exhibit kinase activity, it likely modulates activity of the activated TGFβ receptors by recruiting effector proteins. In support of this theory, endoglin's intracellular domain interacts with zyxin which binds to the intracellular adaptor protein p130Cas (Conley et al., 2004). p130Cas couples ECM/cell interactions to several intracellular signaling pathways and has been shown to be required for some TGFβ1- mediated responses as well as bind the R-SMAD SMAD3 and regulate its phosphorylation and transcriptional activity (Choi et al., 2002; Kim et al., 2008a). Cellular responses to TGFβ1 stimulation depend on the specific ALK receptors expressed by that cell type. Pericytes express ALK5, which phosphorylates SMADS 2 and 3 (Goumans et al., 2009). Activation of ALK5 signaling in these cells inhibits migration and proliferation and induces expression of 1) plasminogen-activator-inhibitor-1 (PAI-1), which itself can interfere with migration, 2) fibronectin, which remains at the interface between the pericyte and quiescent endothelium, and 3) α−SMA, a marker of mature pericytes (Chantrain et al., 2006; Courtoy and Boyles, 1983; Goumans et al., 2009; Sato and Rifkin, 1989; Stefansson and Lawrence, 1996). In addition to ALK5, endothelial cells express

ALK1, which can directly phosphorylate SMADs 1 and 5 (Goumans et al., 2009). These receptors induce opposing responses: activation of ALK1 induces endothelial proliferation and migration, while activation of ALK5 induces growth arrest and quiescence (Goumans et al., 2002; Oh et al., 2000). The response to TGFβ1 is dependent on the relative ratio of receptors, the concentration of TGFβ1 present, as well as on the expression of endoglin, which promotes ALK1 signaling and directly inhibits ALK5 R-SMAD activity (Goumans et al., 2003; Lebrin et al., 2004; Pepper et al., 1993; Plouet and Gospodarowicz, 1989). In addition to activating R-SMAD transcription factors, the activated TGFβ/receptor complex also recruits the mitogen-activated kinase kinase kinase (MAP3K) TGFβ-activated kinase-1 (TAK1), which stimulates phosphorylation and activation of p38. TGFβ1-activated p38 can then induce SMAD-independent TGFβ1-induced responses as well as modulate SMAD activity (Sorrentino et al., 2008; Yu et al., 2002). Interestingly, genetic ablation of TAK1 results in embryonic lethality with a vascular phenotype similar to mice lacking expression of TGFβ1, ALK1, or endoglin, suggesting that TAK1-mediated MAPK signaling is required for the function of TGFβ1 in development of the vasculature (Jadrich et al., 2006). Activated TGFβ1 receptor complexes also activate Erk signaling. Both the ALK receptor and TβRII contain tyrosine residues that are phosphorylated upon receptor activation (Lawler et al., 1994; Lee et al., 2007). These phosphorylated tyrosines serve as a binding site for the adaptor proteins Grb and Shc (Galliher and Schiemann, 2007). The tyrosine kinase activity of the activated receptor complex results in direct phosphorylation of ShcA and induces formation of a

ShcA/Grb/Sos complex that leads to Erk signaling. Activation of the Erk signaling cascade results in activation of Erk1, which directly phosphorylates R-SMADs (Funaba et al., 2002; Kretzschmar et al., 1999; Matsuura et al., 2005).

SPARC and TGFβ1 were first shown to be involved with one another at the level of expression. TGFβ1 was found to induce SPARC expression two-fold in a population of fibroblast-like fetal calvarial cells.(Wrana et al., 1988). Later studies revealed that SPARC expression could be induced by TGFβ1 in a variety of cell types (Ford et al., 1993; Reed et al., 1993; Wrana et al., 1991). SPARC can also regulate expression of TGFβ. Lack of SPARC expression in mouse mesangial cells resulted in decreased TGFβ1 expression; addition of recombinant SPARC restored TGFβ1 expression to wildtype levels. (Francki et al., 1999) The bidirectional regulation of expression between SPARC and TGFβ1 suggest that they function in cooperation with one another. In addition to expression, SPARC also has the capacity to regulate TGFβ1 activity, though the precise mechanism behind this regulation is unclear. Mouse mesangial cells isolated from *SPARC*$^{-/-}$ mice exhibited over 50% reduction in basal SMAD2 phosphorylation compared to cells isolated from *SPARC*$^{+/+}$ mice (Francki et al., 2004). Interestingly, this group found that addition of recombinant SPARC to *SPARC*$^{-/-}$ mesangial cells for 30 minutes was sufficient to increase basal SMAD2 phosphorylation, suggesting that the effect was direct and not via upregulation of TGFβ1 expression. In support of this, recombinant SPARC had a synergistic effect on TGFβ1-induced SMAD2 phosphorylation in *SPARC*$^{+/+}$ cells (Francki et al., 2004). Furthermore, Schiemann et al. (Schiemann et al., 2003) found

that SPARC induced SMAD2 phosphorylation in both endothelial and epithelial cells, an effect that was blocked with a neutralizing TGFβ antibody. In contrast to these results, but in line with our own observations, another study found that SPARC had a negative effect on TGFβ1 activity in vivo and in vitro (Chlenski et al., 2007). Transformed human embryonic kidney cells were transfected with SPARC or an empty vector and used in a mouse xenograft model. Tumors expressing SPARC exhibited a significant decrease in the amount of activated fibroblasts as detected by SMAD2-induced α–SMA expression. The group went on to show that forced expression of SPARC by 293 cells suppressed their ability to induce 3T3 fibroblast expression of α–SMA using cell co-cultures, and that conditioned media from 293 cells expressing SPARC was less able to stimulate α–SMA expression compared to conditioned media from control cells. Using recombinant protein, the group went on to show that these effects were a direct result of SPARC. Incubation with purified TGFβ1 induced α–SMA expression 3T3 cells, an effect that was blocked upon addition of recombinant SPARC. Lastly, the group found that recombinant SPARC blocked TGFβ1-induced phosphorylation of SMAD2 in both 3T3 cells and primary human fibroblasts. Interestingly SPARC also controlled FGF2-induced effects on 3T3 cells as conditioned media form 293 cells expressing SPARC was able to enhance the effect of FGF2-induced migration. These results demonstrate that SPARC can limit TGFβ1 activity in vitro and in vivo (Chlenski et al., 2007). Specifically, they demonstrate the ability of SPARC to block SMAD2 phosphorylation and α–SMA expression. TGFβ1/SMAD2 signaling induces pericyte expression of α–SMA as well

as components of the ECM, and inhibits their migration; therefore TGFβ1 activation must be spatially regulated to prevent premature differentiation (Antonelli-Orlidge et al., 1989a; Antonelli-Orlidge et al., 1989b; Sato and Rifkin, 1989; Sato et al., 1990). The results of Chlenski et al. suggest that SPARC may prevent activation of TGFβ1 signaling in pericytes as they migrate towards nascent endothelial tubes (Fig. 1.2 B). In this model, SPARC also blocks activation of latent TGFβ1 complexes as these are constitutively expressed but only activated upon contact of pericytes with endothelial cells. This model is supported by the capacity of SPARC to promote pericyte recruitment in an orthotopic tumor model (Arnold et al., 2010; Puolakkainen et al., 2004). In contrast, the results of the aforementioned studies suggest that SPARC may induce premature differentiation of pericytes during angiogenesis (Fig. 1.2 C) (Francki et al., 1999; Schiemann et al., 2003). The opposing activities exhibited by SPARC in these studies may be due to differences in the source of SPARC protein. SPARC is differentially glycosylated in different cell types, which may dictate the function of SPARC in various in vitro and in vivo assays (Kaufmann et al., 2004). As SPARCs functional relationship with TGFβ1 is currently unclear, studies examining differences in cell lines exhibiting opposing TGFβ1-dependent responses to SPARC should be done.

Regulation of integrin activity by SPARC

Review of integrins

The resting endothelium consists of quiescent endothelial cells in contact with each other and with pericytes. In between the layer of endothelial cells and pericytes lies a layer of basement membrane, to which both cell types strongly adhere. The basement membrane is rich in laminins, type IV collagen, perlecan, and von Willebrand factor (Hynes, 2007). During angiogenesis, the basement membrane is degraded by MMPs secreted by stimulated endothelial cells and pericytes; this allows for each cell type to mobilize away from the preexisting vessel. As each of these cells migrate away from the preexisting vessel, they encounter the surrounding ECM. The ECM functions both as a physical scaffold, providing structural support to the resting vasculature and, as mentioned earlier, as a growth factor depot. This allows for rapid activation of growth factor signaling that is not dependent on the translation and secretion of nascent protein. In addition, structural constituents of the ECM can directly influence vascular cell behavior. Through these differing mechanisms, the ECM is able to regulate all of the processes necessary for angiogenesis including proliferation, migration, invasion, and vessel stabilization.

The ECM contains a diverse mix of structural proteins including collagens, elastins, laminins, vitronectin, and fibronectin. The dependence of angiogenesis on the structure and function of such proteins has been demonstrated in many mouse models. For example, deletion of the laminin $\alpha 4$ chain results in a compromised microvasculature that leads to severe microhemorrhaging (Patton et al., 2001). Deletion of collagen IV α chains causes basement membrane destabilization which leads to embryonic lethality (Poschl et al., 2004). Deletion of fibronectin results in a

malformed vasculature and also results in embryonic lethality (George et al., 1993). These proteins interact with specific integrin receptors expressed by both pericytes and endothelial cells to control processes such as migration and proliferation. Integrins exist as non-covalently associated, transmembrane glycoprotein heterodimers and mediate both cell-cell and cell-ECM interactions (Davis and Senger, 2005). Each heterodimer contains an α-subunit and a smaller β-subunit. There are currently 18 α-subunits and 8 β-subunits that can associate into at least 24 different heterodimers. These heterodimers mediate both "outside in" signaling, that is, they can communicate that they are bound to the ECM to intracellular proteins, and "inside out" signaling, that is, intracellular signaling events can influence the binding affinity of the integrin for the ECM (Hynes, 2007).

α and β-subunits are both type-I transmembrane glycoproteins and have the same general structure: both contain large extracellular regions with multiple domains, both have single-pass transmembrane domains, and both contain small cytoplasmic tails. α-subunits contain four to five extracellular domains. The 4 membrane proximal domains are shared by all α-subunits. The most distal subunit from the membrane is referred to as the β-propeller domain and is found on nine of the 18 α-subunits (α3, α4, α5, α6, α7, α8, α9, αv, and α_{IIb}). This domain is responsible for forming the substrate binding site, together with domain I (the "inserted" domain) of the β-subunit (Luo et al., 2007). Integrins αD, αE, αL, αM, αX, α1, α2, α10, and α11 each contain an I domain that serves as their substrate binding site. β-subunits contain up to eight domains with the I domain being most distal from the membrane. I domains are the

key domains of the integrin 'head' as they are responsible for most of the substrate-binding activity exhibited by integrins. This domain contains a divalent cation-binding site, where Mg^{2+} or Ca^{2+} is bound to aspartate, serine, and threonine side chains and is able to interact with negatively charged residues within the substrate. This site is referred to as the metal ion-dependent adhesion site (MIDAS) and is required by all known integrins for substrate binding activity. The I domain can exist in at least three distinct conformations that allow it to allosterically regulate substrate affinity: a low-energy closed conformation, an intermediate conformation, and an open conformation (Lee et al., 1995). The open conformation is the highest energy conformation and is stabilized upon substrate interaction. It's believed that interactions between adjacent integrins can stabilize the open conformation in the absence of substrate, which results in up to a 10,000 fold increase in substrate affinity compared to the closed conformation (Shimaoka et al., 2003). Binding of substrate to the I domain ultimately results in conformational changes that signals to the transmembrane and intracellular domains and activate intracellular signaling. These conformational changes result in the integrin heterodimer going from an inactive bent conformation, which is stabilized by interactions between the integrin 'head' with the 'legs' (the membrane proximal domains of the extracellular regions), to an activated/primed, straightened conformation, stabilized by interactions between the 'head' and substrate (Arnaout et al., 2005; Takagi et al., 2002). Furthermore, the interactions that stabilize the bent conformation are weak enough so that low energy disturbances induced by interactions between the intracellular domains with intracellular proteins such as talin

can force the bent conformation to straighten out; this is the basis of 'inside out' signaling (Shattil and Newman, 2004).

Specific integrin heterodimers are able to bind to multiple components of the ECM, and individual components of the ECM are able to bind to multiple integrins. ECM components are able to interact directly with the integrin 'head' domain, an interaction that stabilizes the active conformation of the integrin receptor, but may also interact with the integrin 'legs'. 'Head' interactions are based on small motifs within the ECM substrate; these are usually shared by multiple ECM components; this partly explains the degree of multiplicity of receptor/ ligand interactions (Takagi et al., 2002). Generally, there are at least four distinct structural classes of integrin/ substrate interactions (Humphries et al., 2006). The least characterized of these is the I domain-independent mechanism of interaction between integrins α3β1, α6β1, α7β1, and α6β4 and laminin. The interfaces between these integrins and laminin do not involve the I domain of the β6 or β1 subunit, unlike other integrin laminin receptors. β1 integrins can also heterodimerize with I domain-containing α-subunits α1, α2, α10, and α11. These integrins bind to laminin, fibrillar collagens, and type IV collagen to make up the next class of integrin/ substrate interaction. The head domain of these integrins contains an I domain on each subunit, yet it is the α-subunit domain that confers binding activity (Emsley et al., 2000; Knight et al., 2000). While the mechanism behind laminin interactions is currently unclear, these integrins bind to collagens via the collagen-specific, hydroxyproline-containing GFOGER motif. Another group of integrins interact with an acidic motif with the consensus sequence

L/I-D/E-V/S/T-P-S. These integrins include α4β1, α9β1, α4β7, and αEβ7. D is proposed to coordinate the metal cation in the β-subunit I domain as the motif rests in the cleft between the α β-propeller and the β-subunit 'head' (Humphries et al., 2006). This consensus sequence is found on many resident ECM proteins including fibronectin (Tselepis et al., 1997). αVβ3, αVβ5, αVβ8, αVβ6, αVβ1, α5β1, α8β1, and $\alpha_{IIb}\beta3$ each recognize substrates having an RGD motif. Crystal structures were used to find the mechanism of interaction between this motif and several integrin receptors, where the R binds to the β propeller while D coordinates the metal ion in the MIDAS (Chen et al., 2004; Xiong et al., 2002a; Xiong et al., 2002b). This motif is shared between many components of the ECM, including fibronectin and collagen. Activation of resting or primed integrin heterodimers by these interactions results in lateral clustering of integrins and activation of intracellular signaling events that dictate cellular response to the ECM. These events require recruitment of many different proteins to the cytosolic domains of the activated integrin.

Integrin cytosolic domains are relatively small, the longest being up to 60 residues long (Shattil and Newman, 2004). These domains lack kinase activity and consequently, signaling from ECM-bound integrin receptors requires activation of soluble kinases and recruitment of adaptor proteins. The earliest known intracellular event that follows substrate-induced conformational changes in integrins is activation of Src-family kinases (SFKs). Several integrins have been shown to be constitutively associated with c-src or other SFKs, and evidence suggests that activation of these kinases upon substrate binding is a common first step in intracellular signal

propagation (at least in β3, β2, and β1 integrins) (Arias-Salgado et al., 2003; Ginsberg et al., 2005; Obergfell et al., 2002). The general model of activation is based on studies of $\alpha_{IIb}\beta3$ and αvβ3 (Arias-Salgado et al., 2003). SFKs are associated in an inactive conformation with the β tail. Also present in the inactive complex is c-src tyrosine kinase (Csk), which phosphorylates SFKs at an inhibitory carboxyterminal Y residue, thus keeping SFKs in an inactive conformation. Extracellular binding of substrate by the integrin heterodimer results in allosteric changes that cause dissociation of Csk from the resting complex. Protein tyrosine phosphatase 1B (PTP1B) activity then shifts the equilibrium of SFKs to the active conformation (Bjorge et al., 2000). Integrin ligation also promotes recruitment of actin binding proteins including α-actinin, zyxin, and vinculin and activates focal adhesion kinase (FAK), which can further promote activation of SFKs; this allows for signal propagation to commence. Activated FAK stimulates recruitment of integrin linked kinase (ILK), the ILK/ Nck2 interacting protein PINCH, and the ILK/ actin binding proteins α and β Parvin (Brakebusch and Fassler, 2003)). Activated ILK can directly or indirectly activate integrin effecters such as the serine/threonine kinase Akt (Wickstrom et al., 2010). Activated SFKs and FAK also phosphorylate adaptor proteins including paxillin and $p130^{Cas}$ which ultimately creates docking sites for Grb2 and the Ras guanine exchange factor mSOS, thereby coupling MARK signaling with integrin ligation (Schlaepfer and Hunter, 1998; Schlaepfer et al., 1998). FAK and SFKs also directly stimulate phosphorylation of Y644 on type Iγ phosphatidylinositol phosphate kinase (PIPKIγ) (Ling et al., 2003). This results in binding of talin to

PIPKIγ and translocation to the cell membrane. Membrane-associated PIPKIγ is thought to regulate binding of talin to activated integrins through synthesis of phosphotidylinositol 4,5-bisphosphate (PIP_2). PIP_2 also binds to vinculin, and PIP_2-bound vinculin recruits the actin nucleating Arp2/3 complex, thereby localizing f-actin polymerization and membrane extension to sites of integrin/ substrate interaction (DeMali et al., 2002). Membrane-associated talin also mediates 'inside out' activation of integrins through direct interactions with the β-subunit cytoplasmic tail via its phosphotyrosine-binding (PTB) domain (Critchley, 2004; Wegener et al., 2007). This interaction induces integrin heterodimers to change to their high affinity conformations and serves as positive feedback to increase the number of integrin/ substrate interactions, a process termed focal adhesion formation. Talin also couples growth factor signaling to integrin binding affinity. The small GTPase Rap1 becomes activated in response to many different extracellular stimuli including RTK-interacting growth factors. Activated Rap1 directly interacts with Rap1-GTP interacting adaptor molecule (RIAM) which binds to talin and translocates it to the plasma membrane where it can activate integrins (Lee et al., 2009).

The binding of cells to the ECM via integrins results in profound changes in intracellular signaling. These changes dictate the cellular response to the ECM and/ or growth factors. In the case of angiogenesis, integrin/ ECM interactions are required for vascular cell proliferation and migration, and the organization of these cells into new blood vessels. Control of integrin activity by SPARC, therefore, provides another mechanism through which this matricellular protein may impact angiogenesis.

SPARC was first reported to participate in substrate/ cell interactions in studies using bovine aortic endothelial cells. Addition of SPARC to these cells resulted in a loss of focal adhesions as assayed using interference reflective microscopy (Murphy-Ullrich et al., 1995). SPARC also induced diffusion of vinculin out of focal adhesions and redistribution of actin to the cell periphery. Since these early experiments, SPARC has been shown to regulate the expression, surface level, and activity of many integrins and integrin subunits involved in the angiogenic cascade.

Regulation of α6 integrins

Consistent with early results suggesting that SPARC negatively regulates interactions between cells and their substrates, lens epithelial cells harvested from *SPARC*$^{-/-}$ mice exhibited increased focal adhesion formation and adhered faster than cells isolated from *SPARC*$^{+/+}$ mice (Weaver et al., 2006). Flow cytometry and immunofluorescence staining of fixed cells using an antibody that reacts with α6 integrin heterodimers demonstrated that surface levels of these integrins were greater in cells isolated from *SPARC*$^{-/-}$ mice, suggesting that SPARC negatively regulates the expression or surface translocation of α6 integrin heterodimers, and this results in decreased adhesion and focal adhesion formation. As the α6 subunit interacts with β1 integrins in lens epithelial cells, this group went on assess the effect of SPARC on β1 integrin activity using talin-association as a readout. Forced expression of SPARC in cells isolated from *SPARC*$^{-/-}$ mice induced a reduction in the amount of β1 integrin-

associated talin. In line with this, forced SPARC expression also induced a reduction tyrosine-phosphorylated paxillin. Together, these data suggest that SPARC can control the level of α6 integrins, thus indirectly control α6 integrin-mediated events. In support of this, SPARC decreased expression of the α6 subunit in preadipocytes when added exogenously to cells in vitro (Nie and Sage, 2009).

The α6 integrin subunit heterodimerizes with β1 and β4 integrins in endothelial cells and pericytes to form laminin receptors believed to be important in angiogenesis (Silva et al., 2008). In various in vitro angiogenesis assays, expression of α6β4 was highest at the earliest time points of angiogenesis and decreased overtime (Hiran et al., 2003). In the same study, α6β4 integrin localized specifically to maturing vessels during normal mouse development in vivo. These data suggest that the α6β4 heterodimer has a negative effect on blood vessel formation (Hiran et al., 2003). In disagreement with this proposal, the heterodimer was widely detected in tumor vasculature, and when grown in mice lacking normal β4 integrin, tumor implants exhibited decreased angiogenesis, suggesting that α6β4 promotes tumor angiogenesis (Nikolopoulos et al., 2004). The discrepancy in conclusions may arise from differences in physiological context, as the tumor microenvironment differs greatly from that observed during normal development (Hanahan and Weinberg, 2000). However, in agreement with α6 integrins promoting angiogenesis, endothelial α6β1 has been shown to interact with the ECM-associated molecule CYR61 and can enhance endothelial tube formation in vitro (Leu et al., 2002; Leu et al., 2003). Though the precise function of α6 integrins in angiogenesis is not conclusive, these

integrins do functionally contribute to angiogenesis. As such, control of α6 expression by SPARC likely serves a regulatory role during angiogenesis, though experiments addressing the function of SPARC on α6 integrins expressed by vascular cells still need to be done.

Regulation of β1 integrin

The β1 integrin subunit interacts with α4 and α5 expressed by both endothelial cells and pericytes to form fibronectin receptors (Abraham et al., 2008; Carnevale et al., 2007). It can also associate with α1 or α2 to form collagen receptors, and with α3 or α6 to form laminin receptors (Senger et al., 2002; Silva et al., 2008). Recently, SPARC has been shown to interact directly with integrin β1 (Weaver et al., 2008). Expression of SPARC in cultured mouse lens epithelial cells increased in response to serum deprivation or to tunicamycin treatment, an inducer of endoplasmic reticulum (ER) stress. The group also observed that ER stress induced more reactive oxygen species (ROS) in cells isolated from *SPARC*$^{-/-}$ mice, which led them to investigate whether SPARC was exerting a prosurvival effect. Indeed, the authors found that *SPARC*$^{-/-}$ cells were more susceptible to stress-induced apoptosis compared to their *SPARC*$^{+/+}$ counterparts. ILK is a critical regulator of cell death via its direct phosphorylation of a variety of proteins including Akt (Wickstrom et al., 2010). As SPARC regulates ILK activity in fibroblasts and of its downstream effectors such as Akt in glioma cell lines, the authors investigated the role of ILK activity in SPARCs prosurvival effect and found that activity was induced during both stress responses

and was required for SPARCs prosurvival effect on lens epithelial cells (Barker et al., 2005; Shi et al., 2004; Weaver et al., 2008). ILK specifically interacts with the cytoplasmic domains of β integrins, therefore the group next examined whether SPARC controlled ILK activity by interacting with β1 integrins. Immunoprecipitation and cross-linking experiments revealed that β1 integrins associate in a complex with SPARC and ILK. Interestingly, a β1 integrin-blocking antibody prevented stress-induced SPARC association with β1 integrin and activation of ILK, suggesting that SPARC may modulate ILK activity via its interaction with β1 integrins. The group mapped SPARCs β1-binding site to residues 113-130, a region that contains the angiogenic, Cu^{2+}-binding sequence GHK (Funk and Sage, 1993; Lane et al., 1994; Thompson et al., 1996). These results suggest that SPARC may interact with β1 integrins to regulate cell survival in vivo. β1 integrins are required for normal mouse development past embryonic day E5.5 (Bouvard et al., 2001; Fassler and Meyer, 1995). In the vasculature, these integrins are expressed by endothelial cells and pericytes of the resting vasculature and during angiogenesis (Carnevale et al., 2007; Silva et al., 2008). Specific deletion of β1 integrin in PDGFRβ-expressing pericytes and VSMCs results in their inability to provide adequate blood vessel-coverage and results in widespread vessel hemorrhaging and a selective disadvantage during postnatal life in mice (Abraham et al., 2008). Furthermore, both focal adhesion maturation and actin stress fiber formation were blocked in isolated β1 integrin-null mural cells, as were their adhesion and migration capacities, suggesting that mural

cells require β1 integrins to respond to the surrounding ECM and associate with endothelial tubes during angiogenesis (Abraham 2008). β1 integrins also regulate endothelial cell behavior during the angiogenic cascade (Carlson et al., 2008; Malan et al., 2010). Specific deletion of β1 integrin in Tie1 or Tie2-expressing endothelial and hematopoietic cells results in a disorganized and discontinuous vasculature that causes lethality at embryonic days E11.5 and E10.5, respectively, in mice (Carlson et al., 2008). Evaluation of the effects of the β1 deletion in isolated endothelial cells revealed that expression was required for survival as well as adhesion and migration on laminin and collagen but not to fibronectin. Furthermore, the embryoid body model of angiogenesis revealed that β1 integrin is essential for endothelial cell sprouting and protects against endothelial cell death during angiogenesis. These studies point to a proangiogenic function for β1 integrins expressed by both mural cells and endothelial cells. As SPARC is expressed and secreted by vascular cells during angiogenesis, it seems highly plausible that the mechanism of SPARC-mediated cell survival through the extracellular interaction of SPARCs GHK domain with β1 integrins may positively regulate vascular cell behavior during blood vessel formation. This proposal is supported by in vitro studies using SPARC and GHK-containing SPARC peptides. SPARC and its derivative peptides promoted chick chorioallantoic membrane angiogenesis, bovine aortic endothelial cell tube formation, and endothelial cell proliferation (Funk and Sage, 1993; Lane et al., 1994). Angiogenic GHK-containing peptides were specifically generated by cleavage of SPARC by the angiogenic protease plasmin (Iruela-Arispe et al., 1995). Though a mechanism in which SPARC,

through its GHK domain, induces angiogenesis though interactions with β1 integrins seems likely, experiments specifically addressing the dependency of SPARC-induced angiogenesis on β1 integrin expression by pericytes and endothelial cells are currently lacking. Furthermore, incubation of lens epithelial cells with a β1 integrin-blocking antibody prevented SPARC from associating with β1 integrins (Weaver et al., 2008). Such antibodies prevent the integrin heterodimer from interacting with their respective ECM substrates. That such an antibody could compete with SPARC for β1 integrin binding suggests that SPARC may function to control access of β1 integrin heterodimers to their respective substrates. As these interactions are required for normal angiogenesis, SPARC activity on β1 integrins is likely to be temporally regulated. Indeed, earlier studies found that SPARC protein but not message was highest in newly formed chick chorioallantoic membrane vasculature between days 9 and 15 but dropped afterwards (Iruela-Arispe et al., 1995). The highest levels of SPARC protein spatially overlapped regions of plasmin protease activity, suggesting that plasmin-induced angiogenic SPARC cleavage products are beneficial only in the early phases of new blood vessel formation. Alternatively, SPARC may interact with a complex of surface proteins that includes β1 integrins and growth factor receptors. Engagement of integrins with their ECM substrates results in lateral clustering and focal adhesion formation. Many growth factor receptors, such as VEGF and PDGF receptors, are recruited into these complexes and can enhance focal adhesion formation and signaling, while other receptors, such as TGFβ receptors, seem to antagonize focal complex formation (Conley et al., 2004; Sundberg and Rubin, 1996;

Wang and Fu, 2001; Yamada and Even-Ram, 2002). It has yet to be ruled out that SPARC actually interacts indirectly with β1 integrins by interacting with a growth factor receptor. In this model, SPARC does not compete with β1 integrin substrates; rather, the inhibition of focal adhesion formation induced by the β1 integrin blocking antibody prevents subsequent recruitment of a SPARC-binding growth factor receptor. To date SPARC has not been shown to interact with such a focal adhesion-promoting growth factor receptor; however, most studies addressing SPARC and growth factor receptor interactions have not examined whether SPARC-derived peptides can exhibit such activity. Clearly, studies addressing the formation and constituents of the SPARC/ β1 integrin complex are required to gain a more complete understanding of the relationship between SPARC and β1 integrins.

Regulation of αV integrins

αV integrin receptors represent the most studied group of integrins involved in angiogenesis to date (Hynes, 2007). Genetic ablation of the αV subunit results in perinatal lethality due to abnormal association of cerebral blood vessels with the surrounding parenchyma (McCarty et al., 2002). A plethora of studies have revealed that αV integrin expression in vascular cells in response to various angiogenic stimuli (Brooks et al., 1994; Hynes, 2007). These integrins can dimerize with β3, β5, or β8 subunits to form vitronectin receptors, and with the β6 subunit to form a fibronectin receptor. The majority of reports demonstrate that αVβ3 and αVβ5 are the only αV

integrins expressed by vascular cells, and these are only expressed in endothelial cells however, we and others have found that stellate cells, which function as pericytes in various organs including the pancreas and the liver, express αV, β3, β5, β6, and β8 (Alghisi and Ruegg, 2006; Beer and Schwaiger, 2008; Somanath et al., 2009a; Somanath et al., 2009b). Specific antagonism of αVβ3 and αVβ5 has proven to be an effective strategy for blocking tumor angiogenesis in clinical trials with glioblastoma patients (Desgrosellier and Cheresh, 2010) and studies with of human dental pulp cells and various tumor lines have shown that SPARC is able to modulate expression and activity of both of these intergins.

In a study on bone metastasis of prostate cancer, SPARC was shown to regulate migration of the human prostate cell line LNCaP (De et al., 2003). This group found that bone extracts harvested from *SPARC*$^{-/-}$ mice were less able to induce LNCaP cell migration compared to *SPARC*$^{+/+}$ extracts. Supplementation of *SPARC*$^{-/-}$ bone extract with recombinant SPARC was able to rescue this effect. The stimulatory effect of SPARC required LNCaP αVβ3 and αVβ5, as specific blockade of either of these receptors significantly decreased SPARC-induced migration. Interestingly, blockade of VEGFR2 had a similar effect on SPARC induced migration. Ligation of SPARC by αVβ5 was found to induce VEGF expression, an effect that was blocked with the αvβ5 specific inhibitor cRGDfV. These results suggest that SPARC induces αVβ3 and αVβ5 activity. In line with this conclusion, SPARC induced the migration of human dental pulp cells in a dose-dependent manner (Pavasant et al., 2003). This effect was blocked by treating the cells with the αVβ3 integrin-inhibiting antibody

LM609. In contrast to the activating effect of SPARC on αVβ3 and αVβ5 in human prostate cancer and dental pulp cells, SPARC seems to have a negative effect on these integrins in human ovarian cancer cells. Recombinant SPARC was able to block Mn^{2+}-induced, αVβ3-mediated adhesion of several human ovarian cancer cell lines to vitronectin (Said and Motamed, 2005). The specificity of this effect being mediated by αVβ3 was demonstrated using LM609: SPARC was able to inhibit Mn^{2+}-induced adhesion as much as LM609. Incubation of SKOV3 cells with SPARC decreased surface expression of αVβ3 by 74%, and αVβ5 by 32%, with similar effects in OVCAR3 and IGROV1 cells. Using an antibody that specifically detects αVβ3 in its high affinity conformation, the group went on to show that SPARC reduced the level of activated αVβ3 on the surface of SKOV3 cells. Furthermore, SPARC blocked serum induced FAK phosphorylation. In a follow up study, the same group found that SPARC blocked αV integrin-induced adhesion of ID8 mouse ovarian cancer cells to vitronectin and to peritoneal explants (Said et al., 2007). Altogether, the results obtained using ovarian cancer cell lines suggest that SPARC blocks αVβ3 and αVβ5 activity. This is in stark contradiction to the stimulatory role of SPARC on these integrins suggested by the results of earlier studies (De et al., 2003; Pavasant et al., 2003). The discrepancy suggests that the SPARC controls αV integrin activity in these cell types through some indirect mechanism. One possibility is that SPARC can regulate αV integrin turnover (Fig. 1.3). Integrin turnover is required for many integrin-mediated events including cell migration. In order for cells to migrate, ECM-bound integrins are endocytosed and must be recycled to the cell surface where they

reengage the ECM (Caswell et al., 2009). It may be that SPARC has no effect on activation (though having a positive effect on activation would also fit with this model) or endocytosis of αV integrins in all cell types, but that SPARC blocks recycling of these back to the cell surface in a subset of cell types including ovarian tumor cells. That SPARC decreased surface levels of αVβ3 and αVβ5 in ovarian tumor lines is in line with this hypothesis. Recycling of endocytosed αVβ3 back to focal complexes has been shown to be induced by PDGF-BB-stimulated PDGFR in fibroblasts (Roberts et al., 2001; Vukmirica et al., 2006). PDGFR stimulation results in activation of the Rab4 effectors Rabip4 and Rabip4' which leads to fusion of $\alpha v\beta 3^{+}$ early endosomal vesicles to the cell membrane. If the ovarian cancer cell lines used in the above studies rely on PDGF-B signaling for αvβ3 turnover, then addition of SPARC would inhibit integrin recycling by virtue of its capacity to block PDGF-B from interacting with its receptors. Also, activation of VEGFR2 by VEGF-A has been shown to induce recycling of $VEGFR2^{+}$ $Rab4^{+}$ vesicles to the cell surface in endothelial cells (Gampel et al., 2006). It seems possible that human prostate cancer and dental pulp cells may rely on a VEGF-A/ VEGFR2 feedback loop to recycle endocytosed αVβ3 integrins back to their surfaces. Though VEGFR expression has traditionally been associated with endothelial cells, it is becoming clear that many nonendothelial cell types use these receptors to regulate autocrine signaling (Berthaut et al., 2009; Dias et al., 2001; Lichtenberger et al., 2010; Mercurio et al., 2005). That SPARC promotes αV integrin-mediated migration of these cells by stimulating a VEGF-A/ VEGFR2 feedback loop is supported by previous studies demonstrating that

SPARC selectively blocks VEGF-A/ VEGFR1 interactions, thereby promoting VEGF-A/ VEGFR2 interactions (De et al., 2003; Nozaki et al., 2006). Furthermore, blockade of VEGFR2 and VEGF in the above study prevented SPARC-induced migration of prostate cancer cells (De et al., 2003). Regulation of αVβ3 and αVβ5 trafficking may represent a novel mechanism of SPARC mediated-regulation of vascular cell migration.

αV integrins and TGFβ1

In addition to mobilizing the intracellular machinery in response to ECM, αVβ6 and αVβ8 also activate latent TGFβ1 (Annes et al., 2004; Mu et al., 2002). αVβ6 interacts with the RGD motif of LAP found on the latent TGFβ1 complex. In a mechanism that requires actin polymerization and cell to cell contact, αVβ6 induces a conformational change in the latent complex, exposing the active TGFβ1 dimer without cleavage and making it available to TGFβ receptors on adjacent cells. αVβ8 also interacts with the LAP RGD motif; however, activation of the latent TGFβ1 complex is achieved via MMP14-mediated release of the active TGFβ1 dimer from the latent complex (Sheppard, 2004). As previously mentioned, we have found that pancreatic pericytes express these integrins in vitro; therefore, studies addressing αV integrin-mediated activation of TGFβ1 in pericytes are warranted. As TGFβ1 activity is induced in pericytes upon contact with the endothelium, αV integrin-mediated activation of latent TGFβ1 may provide a mechanism through which these cells

spatially restrict TGFβ1 activity (Antonelli-Orlidge et al., 1989a; Sato and Rifkin, 1989; Sato et al., 1990).

SPARC and TGFβ1: regulation of pericyte behavior

That SPARC is involved in regulating angiogenesis is supported by 1) the induction of SPARC expression by the vasculature during both physiological and pathological angiogenesis, 2) its ability to modulate activity of growth factors required for angiogenesis, 3) its ability to regulate expression and activity of MMPs required for angiogenesis, and, 4) by its ability to regulate integrins that are utilized by endothelial cells and pericytes during angiogenesis. For these reasons, modulation of SPARC activity may prove to be a beneficial strategy against diseases characterized by blood vessel formation. Perhaps the most immediately relevant of such diseases is cancer (Carmeliet and Jain, 2000). Tumor-associated blood vessels are typically quite different from the vasculature of normal tissues. They are tortuous, leaky, and are often discontinuous, characteristic features of an unstable vasculature, and are comprised of abnormal endothelial cells and pericytes (Carmeliet and Jain, 2000). Solid tumors require new blood vessel formation for their growth beyond 2 mm^3, therefore anti-angiogenic agents are a useful therapy for treating such diseases (Ferrara, 2002; Folkman, 1971; Hanahan and Weinberg, 2000). The resulting tumor vasculature contains a mixture of vessels with varying degrees of pericyte coverage. Pericytes within the tumor vasculature exhibit increased expression of α–SMA, are loosely associated with the endothelium, extend membrane processes into the tumor

tissue, and survive anti-VEGF-A therapy (Baluk et al., 2005; Morikawa et al., 2002). Tumor associated pericytes also provide survival factors to their associated endothelium, allowing vessels to survive in the face of anti-VEGF-A therapy (Gerhardt and Semb, 2008). It has already been established that pericytes represent a beneficial target for anti-angiogenic therapy. Targeting pericytes by blocking PDGFRβ enhanced the ability of anti-VEGF-A therapy to decreased tumor blood vessel number and control tumor growth in a mouse model of islet carcinoma (Bergers et al., 2003). SPARC promoted pericyte recruitment in orthotopic pancreatic tumors grown in mice, suggesting that SPARCs ability to block PDGF-B from binding to PDGFRβ is not dictating its effect on pericyte behavior (Puolakkainen et al., 2003). Currently, studies demonstrating the inhibitory activity of SPARC on PDGF-B are limited to 1 report demonstrating interaction of purified SPARC with purified PDGFB and another demonstrating that SPARC blocks the proliferative effect of PDGF-B on arterial smooth muscle cells (Motamed et al., 2002; Raines et al., 1992). More experimental evidence suggests that SPARC may control TGFβ1-mediated responses in vivo (Chlenski et al., 2007; Ford et al., 1993; Francki et al., 1999; Francki et al., 2004; Reed et al., 1993; Schiemann et al., 2003; Wrana et al., 1988; Wrana et al., 1991)(Wrana 1988, Wrana 1991, Ford 1993, Reed 1993, Francki 1999, Francki 2004, Schiemann 2003, Chlenski 2007). These studies show that SPARC can both promote and inhibit TGFβ1 activity. Regulation TGFβ1 activity in pericytes by SPARC may represent a novel mechanism for modulating pericyte behavior in vivo with clinical implications in tumor therapy.

To elucidate the effect of SPARC on pericyte behavior, we first determined that SPARC was indeed express by pericytes in vivo. Using a genetic model of pancreatic ductal adenocarcinoma, we found that SPARC expression correlated with more pericyte-associated tumor vessel. We then isolated primary pericytes from pancreata of *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ mice. Pericytes isolated from *SPARC*$^{-/-}$ pancreata induced significantly fewer tubes and less branching of bEnd.3 endothelial cells in an in vitro tube formation assay. To assess the effect of SPARC on pericyte migration, we first determined whether SPARC controlled fibronectin mediated focal adhesion formation. *SPARC*$^{-/-}$ pericytes exhibited significantly fewer vinculin-tipped filopodia while spreading than their *SPARC*$^{+/+}$ counterparts. In contrast, vinculin was seen in large focal complexes surrounding the cell periphery. Focal adhesions were then visualized in fully adhered pericytes. Vinculin^{+}/f-actin^{+} focal adhesions were significantly larger in *SPARC*$^{-/-}$ pericytes, though *SPARC*$^{+/+}$ pericytes exhibited a more of these structures. Using a modified Boyden chamber assay, we found that *SPARC*$^{-/-}$ pericytes were less able to migrate towards fibronectin than their *SPARC*$^{+/+}$ counterparts; this effect was blocked when treating *SPARC*$^{-/-}$ cells with a TGFβ-neutralizing antibody an effect that was also seen when targeting SPARC with shRNA or with a neutralizing antibody in 10T1/2 cells. We also found that *SPARC*$^{-/-}$ pericytes exhibited increased SMAD2 phosphorylation and *PAI-1* expression. Using co-immunoprecipitation, solid-phase binding assays, and immunofluorescent staining of primary pericytes, we found that SPARC interacts with the TGFβ1 accessory receptor endoglin. We then found that knock down of endoglin reversed the inhibitory effect of

targeting SPARC on pericyte migration. Examination of endoglin localization in pericytes revealed that endoglin aberrantly associated with vinculin in focal complexes of spreading cells in the absence of SPARC. As αV integrins can mediate activation of TGFβ1, we next assessed whether these integrins were controlling TGFβ1 activity in pericytes. Incubation of *SPARC*$^{-/-}$ pericytes with a pan-αV integrin blocking antibody abolished the increase in SMAD2 activity exhibited by *SPARC*$^{-/-}$ pericytes and had no effect on *SPARC*$^{+/+}$ SMAD2 phosphorylation. Together, these data demonstrate that SPARC is able to control pericyte behavior and suggest that SPARC promotes pericyte migration by limiting TGFβ1 activity.

Figure legends

Figure 1.1. **The quiescent microvasculature in adult mouse pancreas**. Arteriole, venule, and capillary endothelial cells (green) are invested with pericytes (red). Note the proximity of pericyte and capillary endothelial cell nuclei in the panels on the right. Also note how the pericyte membrane wraps the around the capillary. Frozen pancreas was sectioned and stained using antibodies against MECA32 (green) and NG2 (red) and visualized using indirect immunofluorescence. Nuclei were visualized using DAPI.

Figure 1.2. **Possible roles of SPARC in controlling TGFβ1-mediated pericyte recruitment during tumor angiogenesis**. (A) Angiogenic cues stimulate pericyte detachment and proliferation. Endothelial cells migrate, proliferate, coalesce, and form a lumen. (B) Pericytes migrate to the newly formed vessel. Upon contact with the endothelium, TGFβ1 is activated in both pericytes and endothelial cells. This induces secretion of ECM components and differentiation. Activation of TGFβ1 inhibits pericyte migration, therefore this step is spatially regulated. Results of Puolakkainen et al. 2004 and Chlenski et al. 2007 suggest that SPARC acts to block premature activation of TGFβ1 in vivo. (C) Results of Francki et al. 2004 and Schiemann et al. 2003 suggest that the presence of SPARC in the angiogenic microenvironment activates TGFβ1. This would result in premature TGFβ1-mediated pericyte differentiation and prevent pericyte recruitment during tumor angiogenesis, in contrast to results of Puolakkainen et al. 2004. PC = pericyte.

Figure 1.3. Proposed **model of regulation of αVβ3 integrin activity by SPARC**. Reports by De et al. 2006, Pavasant 2007, Said et al. 2006, and Said et al. 2007 demonstrate that SPARC can both activate and inhibit αvβ3 activity. 1) αVβ3 heterodimers transform from closed to open confirmations upon ligation with the ECM . 2) This induces their endocytosis. 3) αVβ3 intergrins are stored in a pool of $Rab4^{+}$/$EEA\text{-}1^{+}$ early endosomes in their high affinity conformations. 4) VEGFR2 and PDGFR signaling induces rapid Rab4-dependent recycling to the cell surface where 5) αVβ3 integrins reengage the ECM to facilitate migration. In this model, recycling of αVβ3 is dependent on the growth factor receptors present on the cell surface and on the growth factors in the microenvironment. SPARC promotes αVβ3 recycling in cells expressing VEGFR2 as it increases VEGF-A bioavailability for this receptor, and inhibits αVβ3 recycling in cells reliant on PDGFB as it blocks PDGFB from interacting with its surface receptor.

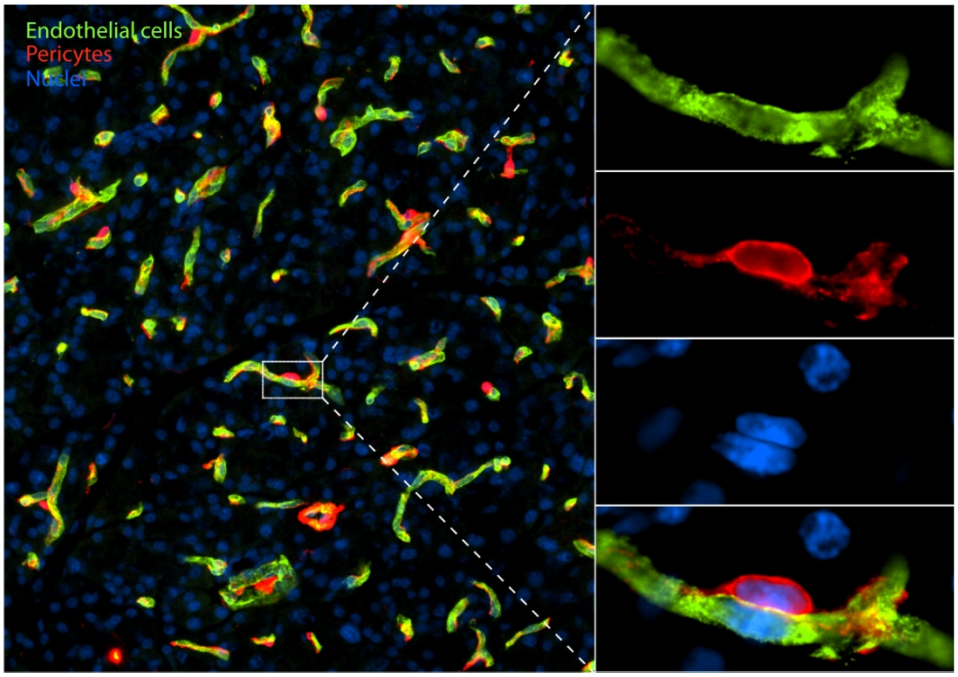


FIGURE 1.1

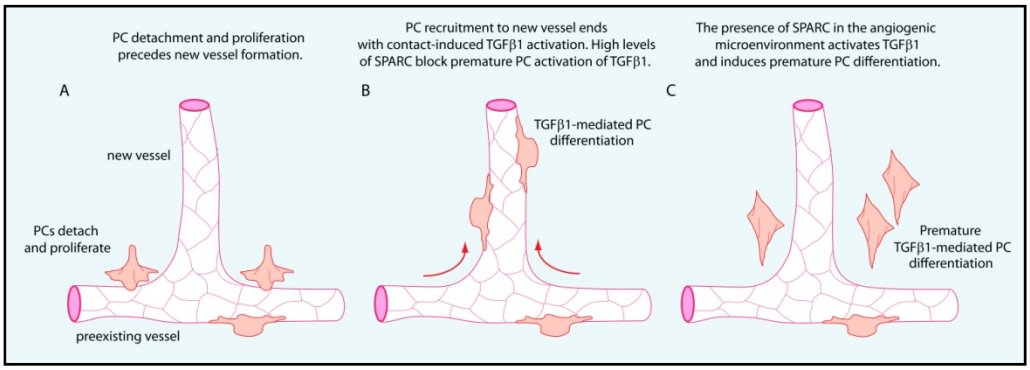

PC detachment and proliferation precedes new vessel formation.
PC recruitment to new vessel ends with contact-induced TGFβ1 activation. High levels of SPARC block premature PC activation of TGFβ1.
The presence of SPARC in the angiogenic microenvironment activates TGFβ1 and induces premature PC differentiation.
A
B
C
new vessel
PCs detach and proliferate
preexisting vessel
TGFβ1-mediated PC differentiation
Premature TGFβ1-mediated PC differentiation

FIGURE 1.2

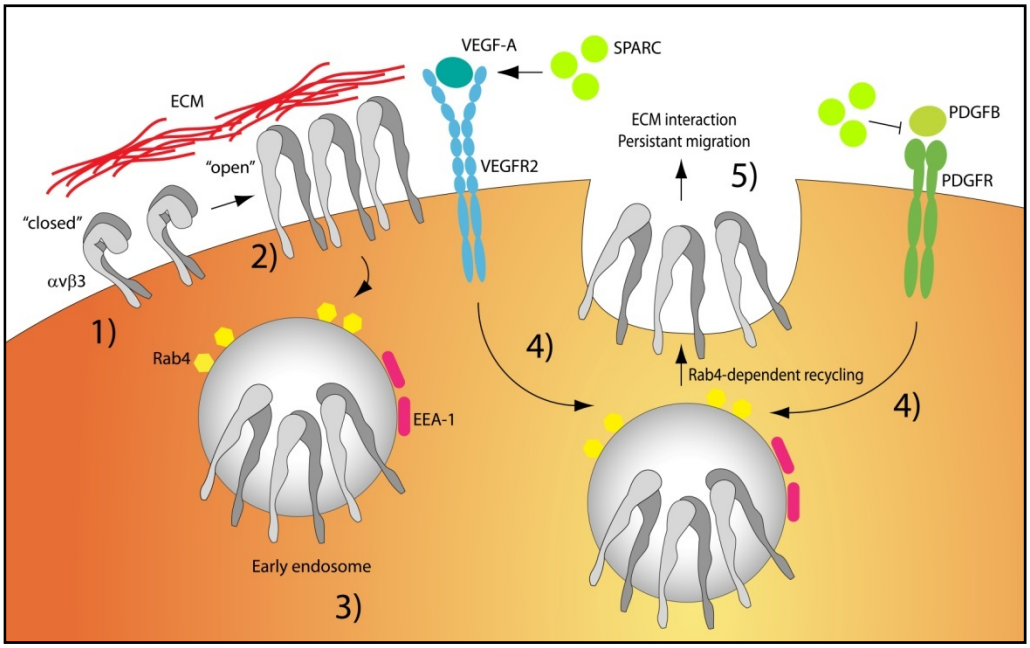

VEGF-A
SPARC
ECM
"open"
"closed"
αvβ3
VEGFR2
ECM interaction
Persistant migration
5)
PDGFB
PDGFR
2)
1)
Rab4
4)
Rab4-dependent recycling
4)
EEA-1
Early endosome
3)

FIGURE 1.3

CHAPTER 2

Regulation of pericyte migration by SPARC

Introduction

During angiogenesis, nascent blood vessels initially form as endothelial tubes that later become coated with pericytes. Pericytes are mobilized from the preexisting vessel by the combined activities of several proliferation and migration-stimulating factors including matrix metalloprotease 9 (MMP9) and PDGF-BB. Mobilized pericytes then migrate to the newly formed endothelial tube, and upon contact, induce vessel maturation and stabilization. This recruitment of pericytes is a required step for normal angiogenesis as lack of adequate pericyte coverage results in vessel abnormalities including excessive leakiness and microhemorrhaging (Hellstrom et al., 2001; Lindahl et al., 1997). Unlike normal vasculature, vessels within tumors are typically leaky, tortuous, and exhibit abnormal pericyte coverage (Benjamin et al., 1999; Eberhard et al., 2000; Helmlinger et al., 1997). Anti-angiogenic tumor therapy is believed to be effective at treating some types of human cancer by selectively ablating vessels that lack pericyte coverage, thereby increasing the efficiency of blood transport within the tumor, which increases delivery of chemotherapeutics (Gerhardt and Semb, 2008). Recently, targeting pericyte recruitment was shown to increase the efficacy of anti-angiogenic tumor therapy in a mouse model of islet carcinoma (Song et al., 2005), demonstrating that modulation of pericyte behavior can be clinically beneficial. Further development of such approaches, however, requires a better understanding of the biological factors that control pericyte behavior.

Secreted Protein Acidic and Rich in Cysteine (SPARC) is matricellular protein whose expression is induced during angiogenesis (Iruela-Arispe et al., 1995;

Lane et al., 1994). SPARC has been implicated in the regulation of a variety of cellular processes critical to angiogenesis including migration, proliferation, and differentiation (Chlenski et al., 2007; Francki et al., 2003; Kupprion et al., 1998; Motamed et al., 2003). This regulatory activity of SPARC seems to be indirect and relies in part on its ability to influence various growth factor signaling pathways. For example, SPARC can directly interact with vascular endothelial growth factor-A (VEGF-A) and with platelet-derived growth factor-BB and AB (PDGF-BB and PDGF-AB) and prevent their interaction with vascular endothelial growth factor receptor 1 (VEGFR1) and platelet-derived growth factor receptor β (PDGFRβ) respectively, while it antagonizes fibroblast growth factor 2 (FGF2)/fibroblast growth factor receptor 1 (FGFR1) signaling without interacting with either the soluble growth factor or the receptor (Hasselaar and Sage, 1992; Kupprion et al., 1998; Motamed et al., 2003). In addition to controlling growth factor signaling, SPARC also interacts with components of the ECM and their receptors to modulate the interaction between cells and their substratum (Bradshaw, 2009; Murphy-Ullrich et al., 1995; Weaver et al., 2008). Using an orthotopic model of pancreatic ductal adenocarcinoma (PDAC), we previously found that both vascular function and pericyte recruitment were decreased in SPARC-deficient mice (Arnold et al.; Puolakkainen et al., 2004). Though the mechanism underlying these results is unknown, they suggest that SPARC positively regulates pericyte behavior in vivo.

Transforming growth factor β1 (TGFβ1) is a pleiotrophic cytokine expressed by endothelial cells and pericytes during angiogenesis. TGFβ1 is secreted in a latent

complex which must be cleaved or otherwise manipulated to expose the active protein (Annes et al., 2004; Derynck et al., 1986; Munger et al., 1999). The precise cellular responses induced by active TGFβ1 depend on both the specific TGFβ receptors expressed and the amount of receptors expressed (Schmierer and Hill, 2007). These responses vary and may include either stimulation of migration, inhibition of migration, apoptosis, or proliferation (Daly et al., 2008; Goumans et al., 2003; Guasch et al., 2007; Yamashita et al., 2008). Receptors to active TGFβ1 are ubiquitous throughout all tissues; therefore both the activation of latent TGFβ1 and the bioavailablity of active TGFβ1 are tightly regulated in vivo (Imai et al., 1997; Lyons et al., 1988; Saharinen et al., 1999). Active TGFβ1 inhibits pericyte migration and induces expression and secretion of basement membrane proteins; therefore activation of TGFβ1 is spatially restricted during angiogenesis, occurring only upon contact of mobilized pericytes with the endothelial cells of the newly formed vessel (Kojima et al., 1991; Owens, 1995; Sato and Rifkin, 1989). The mechanisms that spatially regulate this activation are unclear**.** Recently, SPARC was shown to prevent activation of TGFβ1 in a mouse xenograft tumor model (Chlenski et al., 2007). This group also found that SPARC was able to inhibit TGFβ1 activity in fibroblasts in vitro. Specifically, SPARC blocked expression of α-smooth muscle actin (α-SMA) and phosphorylation of SMAD2.

In the current study, we sought to find whether SPARC regulates pericyte behavior. As SPARC can regulate TGFβ1-mediated processes in vivo and in vitro, we examined the possibility that SPARC might regulate pericyte behavior by controlling

TGFβ1 activity. We report here that SPARC is required for normal pericyte migration. We found that SPARC is expressed by pericytes in the resting vasculature of adult pancreas and in PDAC. We found that SPARC-deficiency in primary pericytes results in increased basal TGFβ1 activity and decreased cell migration, an effect blocked by inhibiting TGFβ1. Furthermore; we show that SPARC interacts directly with the TGFβ1 accessory receptor endoglin. Endoglin aberrantly associates with focal complexes in SPARC-deficient pericytes. SPARC-deficiency results in endoglin-mediated blockade of pericyte migration and TGFβ1 signaling, an increase in αV integrins present in endoglin immunoprecipation complexes, and αV integrin-mediated activation of TGFβ1. These results demonstrate SPARC controls pericyte migration by preventing endoglin from interacting with αV integrins, thereby repressing TGFβ1 activity. A role for SPARC in regulating pericyte recruitment highlights it as a potential target for diseases characterized by abnormal pericyte behavior.

Materials and Methods

Animal husbandry

PDAC (*P48Cre*$^{+}$*:LSLKrasG12D*$^{+}$*:INK4A*$^{lox/lox}$) mice were crossed with *SPARC*$^{+/+}$ or *SPARC*$^{-/-}$ mice to produce *wild type PDAC* (*WT PDAC*) or *SPARC-null PDAC* (*KO PDAC*) mice. For tumor analyses, mice were sacrificed once becoming moribund, with at least 6 mice per group. Tumors were preserved in formalin or snap frozen using liquid nitrogen.

Antibodies

Antibodies to the following proteins were used for indirect immunofluorescent microscopy where indicated: α-SMA (NeoMarkers), NG2 (AB5320 Millipore), desmin (Ab907 Chemicon), vinculin (V4139 Sigma), MECA32, SPARC (R&D), and endoglin (MJ718). For function blocking assays: Pan-TGFβ-neutralizing antibody was purchased from R&D (1D11), SPARC-neutralizing antibody (mAb 293), αVβ6-blocking antibody was a gift from Dean Sheppard (University of California, San Francisco), and αV integrin-blocking antibody was purchased from Biolegend (RMV-7). For western blots: SPARC (clone 303), ALK5 (SC-398 Santa Cruz), TβRII (SC-220 Santa Cruz), endoglin (clone MJ718), αV integrin (AB1930 Chemicon), FAK (3285 Cell Signaling), phosphoSMAD2 (AB3849 serine 465/467, Chemicon), and total SMAD2 (3107 Cell Signaling). For solid phase binding assays: endoglin (MJ718), and SPARC (mAb 236). For immunoprecipitations: SPARC (clone 303), ALK5 (Santa Cruz), TβRII (SC-220 Santa Cruz), and endoglin (MJ718).

Primary pericyte isolation, cell culture, and transfections

Mouse pancreata from four week old *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ mice were minced then subjected to digestion with 1% collagenase type 1, 1X Dulbecco's Modified Eagles Medium (DMEM), 10 mM HEPES, 1% fetal bovine serum, PBS at 37^{o} C until a single cell suspension was obtained. Cell suspensions were centrifuged at

low speed to pellet large debris, resuspended in wash buffer, and passed through a 70 um cell strainer. The resulting cell suspension was then incubated with sheep anti-rabbit IgG-conjugated magnetic Dynabeads (Invitrogen) and rabbit anti-NG2 IgG (Millipore) at 4°C. Dynabeads were preincubated with anti-NG2 IgG overnight at 4° C on a nutator then washed 3 times in wash buffer to remove NaN_2. Bead-bound cells were separated from unbound cells using a BD IMagnet (BD). Primary pericytes were maintained in 10% fetal bovine serum-supplemented DMEM and used between passage 1 and 7 for experiments. 10T1/2 cells were used before 10 passages and maintained in 10% fetal bovine serum-supplemented DMEM. Primary pericytes were transfected using Lipofectamine 2000 (Invitrogen) while 10T1/2 cells were transfected using Fugene (Roche). For shRNA knockdown of SPARC, endoglin, and TGFβ1, shRNA-expression plasmids were purchased from Sigma Mission. 2 ug of plasmid DNA was mixed with 3 ul of transfection reagent and used to transfect 150,000 cells. Cells were used 48 hours post transfection. For negative control transfections, a non-targeting shRNA expression plasmid was used (Sigma Mission).

Cord formation assay

10,000 bEnd.3 endothelial cells were plated onto matrigel-coated three-well chamber slides (BD) in the presence or absence of either 10,000 *SPARC*$^{+/+}$ or *SPARC*$^{-/-}$ pericytes and allowed to self assemble into cords for 17 hours in DMEM supplemented with 0.75% fetal bovine serum at 37° C before visualization by fluorescent microscopy. Before use in the assays, bEnd.3 cells and pericytes were

stained with either the red fluorochrome PKH26, or the green fluorochrome PKH67, respectively (Sigma). Experiments were done three times and in triplicate. Images were taken at 4X magnification, with five images taken per well. The peripheral zone of matrigel was avoided during image acquisition so to avoid cord artifacts associated with changes in surface elevation. Cord lengths and widths were calculated using Elements software (Nikon). For cord width measurements, widths were taken halfway into the length of each cord.

Transwell assay

Ttranswell inserts with 8 um pores were used for migration assays. Inserts were placed in 24-well tissue culture plates for the duration of experiments. The bottom sides of the insert membranes were coated with 10 ul of 1 ug/ul fibronectin for 1 hour at 37^{o} C. Inserts were then used immediately for experiments (Sigma). 7,000 or 5,000 primary pericytes or 10T1/2 cells were added into the insert reservoir in DMEM in a total volume of 125 ul, while DMEM containing 0.1% fetal bovine serum was added into the tissue culture plate well. Experimental conditions were always added to both the top and bottom of the transwell. Cells were allowed to migrate to the fibronectin-coated side of the insert membrane for 6 hours. Cells on the non-coated side of the insert membrane were removed; cells that migrated to the underside of the membrane were fixed in formalin and manually counted. Experiments were done in triplicate and repeated two or three times as indicated.

Scratch assay

Pericytes were seeded onto fibronectin coated wells of a 96-well tissue culture plate at confluency. Scratches were then introduced and images were capture at 0 and 24 hours. The denuded area was measured at these time points using Elements software. Area of migration was calculated as the difference in denuded area at 0 hours and 24 hours. Area of migration was normalized to cell viability, which was measured using the CellTiter Blu assay according to insert instructions (Promega). Values presented represent ((area of migration)/(cell viability)) of each condition normalized to ((area of migration)/(cell viability)) of the cells alone condition. Assays were done in quadruplet and repeated three times.

TGFβ1 ELISA and TGFβ1-response gene express expression

TGFβ1 ELISA kit that detects the active form of TGFβ1 was purchased from Promega. Sample preparation for active TGFβ1 ELISA: primary pericytes were seeded at 100,000 cells per well in 6-well tissue culture plates and cultured in 0.75% fetal bovine serum-supplemented DMEM in triplicate. Cells did not exhibit any expansion and remained subconfluent and viable for the duration of the experiment. Conditioned media and cell lysates were collected at 24, 48, 72, and 96 hours post seeding. M-Per cell lysis buffer supplement with 1X Complete Mini protease inhibitor was used for lysates preparation (Roche). ELISA was performed according to kit instructions. Active TGFβ1 Concentrations were calculated by interpolating values onto a standard curve generated with TGFβ1 accompanying the kit. For qPCR

expression analyses, fold change was calculated using delta delta Ct method where *WT* at 0 pg/ml was the reference sample and *GAPDH* was the reference gene. Sample preparation for qPCR expression analyses: serum starved primary pericytes were seeded at 100,000 cells per well in fibronectin-coated 6-well tissue culture plates in triplicate. Active TGFβ1 purchased from Peprotech was added to serum starved pericytes at final concentrations of 0, 50, and 5000 pg/ml. Cells were then incubated for 17 hours at 37^{o} C. RNA was harvested using TRIZOL reagent (Sigma). cDNA was synthesized using iScript (BioRad). 12.5 ng of cDNA was used per 96-well PCR plate well, with each tissue culture plate well represented in 3 individual PCR plate wells. The following primer sets were used for qPCR or reverse transcription PCR (where indicated): *CTGF*: *CTGF* forward 5' AGC CTC AAA CTC CAA ACA CC 3', *CTFG* reverse 5' CAA CAG GGA TTT GAC CAC 3'; *PAI-1*: *PAI-1* forward 5' GAC ACC CTC AGC ATG TTC ATC 3', *PAI-1* reverse 5' AGG GTT GCA CTA AAC ATG TCA G 3'; *BIGH3*: *BIGH3* forward 5' TGA TAA GAG GGG ACG GTT TG 3', *BIGH3* reverse 5' ATT GGT GGG AGC AAA AAC AG 3'; *GAPDH*: *GAPDH* forward 5' AGA AGG CTG GGG CTC ATT TG 3', *GAPDH* reverse 5' AGG TCG GAG TCA ACG GAT TTG 3'.

Reverse transcription PCR

Cells were incubated in 0.75% fetal bovine serum-supplemented DMEM overnight before RNA extraction and cDNA synthesis. The following primer sets were used for reverse transcription PCR: *endoglin* (L-endoglin): *endoglin* forward 5'

GCA CTC TGG TAC ATC TAT TCT CAC ACA CGT GG 3', *endoglin* reverse 5' GGG CAC TAC GCC ATG CTG CTG GTG G 3'. *SPARC*: *SPARC* forward 5' CTG CGT GTG AAG AAG ATC CA 3', *SPARC* reverse 3' TGG GAC AGG TAC CCA TCA AT 3'; ALK5: ALK5 forward 5' GGC GAC GGC ATT ACA GTG TT 3', ALK5 reverse 5' TGT ACA TAC AAA TGG CCT GT 3'; *TβRII*: *TβRII* forward 5' GCA AGT TTT GCG ATG TGA GA 3', *TβRII* reverse 5' GGT ATC TTC CAG AGT TGA AGC 3'; *TGFβ1*: *TGFβ1* forward 5' TTG CTT CAG CTC CCA CAG AGA 3', *TGFβ1* reverse 5' TGG TTG TAG AGG GCA AGG AC 3'; αV integrin: *itgav* forward 5' GGG TGA TCA TCT TGG CAG TT 3', *itgav* reverse 5' GAA CTT GGA GCG GAC AGA AG 3'; β6 integrin: *itgb6* forward 5' CCG GCT GGC CAA AGA GAT GT 3', *itgb6* reverse 5' AGT TAA TGG CAA AAT GTG CT 3'.

Detection of basal SMAD2 phosphorylation

Pericytes were seeded at 100,000 cells per well of 6-well culture plates and cultured in 1.5% fetal bovine serum-supplemented DMEM for 17 hours before being lysed in sample buffer 300 ul sample buffer: 62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% SDS, 10% glycerol, 50 mM DTT, 0.01% bromophenol blue. Lysates were subjected to SDS-PAGE immediately thereafter. For experiments assessing the contribution of αVβ6 integrin with 10D5, cells were incubated in the presence on 1 mM $MgCl_2$

Immunoprecipitation

10T1/2 cells were lysed in modified RIPA buffer: 0.5% deoxycholate, 0.5% SDS, 1% Triton X-100, 10 mM sodium phosphate pH 7.2, 150 mM sodium chloride, 1X Complete Mini protease inhibitor. Pericytes were lysed in a milder buffer containing 1% NP40, 10 mM sodium phosphate pH 7.2, and 150 mM sodium chloride, 1X Complete Mini protease inhibitor. Lysis was performed on serum starved adherent cells after washing with chilled PBS. Cells were scraped using 1 ml modified RIPA buffer. Lysates were allowed to rotate at 4^{o} C on a nutator for 1 hour, then vortexed several times before centrifuging at 13,000 r.p.m. for 10 minutes to pellet any insoluble material. Lysates were then precleared with protein A/G beads (Pierce). 200 ug of cellular protein in 1 ml lysis buffer was used per immunoprecipitation reaction. 1ug of the appropriate IgG was added with 20 ul protein A/G bead slurry to each sample; each sample was then allowed to rotate overnight at 4^{o} C on a nutator. Immunoprecipitated complexes were washed twice in lysis buffer, then boiled in sample buffer and subjected to SDS-PAGE and western blot analysis.

Solid phase binding assays

Wells of 96-well clear well assay plates were coated with recombinant human SPARC, recombinant human endoglin (R&D), or serum (EastCoast Bio), blocked, and incubated with recombinant endoglin or recombinant SPARC. Bound endoglin or SPARC was detected with anti-endoglin (MJ718) or anti-SPARC (mAb 303) antibodies, or and detected with horseradish peroxidase-conjugated secondary IgG.

Assays were developed using TMB reagent (Pierce). Samples were added in triplicate and experiment was repeated three times.

Surface protein labeling

Primary pericytes were grown to 80% confluency then switched to 0.75% fetal bovine serum-supplemented DMEM. Cells were then labeled with Sulfo-NHS-SS-Biotin Cell Surface Protein Isolation according to supplied instructions (Pierce). 4-10 cm dishes per pericyte genotype were used per fractionation. Fractionations were done twice.

Results

SPARC deficiency results in defective pericyte recruitment in vivo and impaired pericyte migration in vitro

Previously, we found that orthotopic pancreatic tumors grown in *SPARC*$^{-/-}$ mice contained significantly fewer α-smooth muscle actin (α-SMA)$^{+}$ cell-invested blood vessels compared to their *SPARC*$^{+/+}$ counterparts (Arnold et al.; Puolakkainen et al., 2004). As α-SMA is expressed by only a subset of pericytes, we sought to confirm this finding using the more general pericyte marker NG2 (Crisan et al., 2008). NG2 is expressed by resident pericytes associated with MECA32^{+} blood endothelial cells of normal adult pancreas (Fig. 2.1 A). NG2^{+} cells were found to express SPARC in the vasculature of both normal adult pancreas as well as in pancreatic ductal adenocarcinoma (Fig. 2.1, B and C, respectively). We crossed *P48Cre: LSLKras*^{G12D}*:*

INK4A$^{lox/lox}$ (PDAC) mice, which develop pancreatic ductal adenocarcinoma (Aguirre et al., 2003), to *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ mice. Comparison of NG2^{+} cell recruitment between *SPARC*$^{+/+}$ PDAC and *SPARC*$^{-/-}$ PDAC mice revealed fewer pericyte-associated MECA32^{+} vessels in *SPARC*$^{-/-}$ PDAC tumors, confirming previous results (Fig. 2.1 F). We next asked whether SPARC regulated the behavior of primary pericytes in vitro. We used anti-NG2 immunomagnetic bead separation to purify pericytes from *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pancreas digests. Purified primary cells expressed pericyte markers and induced bEnd.3 endothelial cell cord formation (Fig. 2.1 G). Analysis of bEnd.3 cord parameters revealed that *SPARC*$^{-/-}$ pericytes induced fewer cords; however, these cords were on average wider than *SPARC*$^{+/+}$ pericyte-induced cords. To investigate the disparity in pericyte function further, we assessed focal adhesion formation in primary *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes. We found that *SPARC*$^{+/+}$ pericytes exhibited ~5 fold more filopodia per cell compared to *SPARC*$^{-/-}$ pericytes when spreading on fibronectin (Fig. 2.2 A). Focal adhesion area was also greater in *SPARC*$^{-/-}$ pericytes, while the overall number was decreased (Fig. 2.2 B). We next assayed migration towards fibronectin using a transwell assay. *SPARC*$^{-/-}$ pericytes exhibited a significantly reduced capacity to migrate, a feature that was reversed by addition of recombinant SPARC to the assay medium (Fig. 2.2 C). Thus, SPARC seems to mediate pericyte recruitment to blood vessels, at least in part, by regulating pericyte migration.

Endogenous TGFβ1 blocks migration of SPARC deficient pericytes

SPARC has been shown to regulate TGFβ1 activity in other cell types (Chlenski et al., 2007; Francki et al., 2004; Schiemann et al., 2003). As TGFβ1 acts as a negative regulator of pericyte recruitment, we asked whether TGFβ1 was involved in the impaired migration of *SPARC*$^{-/-}$ pericytes. To this end, we compared migration towards platelet derived growth factor to migration in the presence of a TGFβ-neutralizing antibody using a transwell assay. Surprisingly, the TGFβ-neutralizing antibody enhanced migration of *SPARC*$^{-/-}$ pericytes to the same extent as did PDGFB, while having no effect on *SPARC*$^{+/+}$ pericyte migration (Fig. 2.3 A). The ALK5 inhibitor SB431542 had a similar effect on *SPARC*$^{-/-}$ pericyte migration (Fig. 2.3 B). Enhanced migration of *SPARC*$^{-/-}$ pericytes in the face of TGFβ inhibition was also observed using a scratch assay (Fig. 2.3 C). To assess whether SPARC directly regulates pericyte migration, we used shRNA to knockdown SPARC expression in pericyte-like 10T1/2 cells. Knockdown of SPARC impaired 10T1/2 cell migration towards fibronectin in the transwell assay (Fig. 2.3 D). Interestingly, the effect of SPARC knockdown was not observed when cells were allowed to migrate in the presence of the TGFβ-neutralizing antibody. Furthermore, migration of 10T1/2 cells was impaired in the presence of a monoclonal anti-SPARC antibody, an effect that was not observed when the anti-SPARC antibody was used in combination with the anti-TGFβ antibody (Fig. 2.3 E). We hypothesized that increased TGFβ1 expression in *SPARC*$^{-/-}$ pericytes was responsible for TGFβ-mediated inhibition of migration in the absence of SPARC. Surprisingly, analysis of total cellular and secreted TGFβ1 revealed no differences between *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes (Fig. 4 A). Reverse

transcription PCR revealed no difference in TGFβ1 expression between *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes (Fig. 2.4 B). Surprisingly, *SPARC*$^{-/-}$ pericytes retained more TGFβ1 on the extracellular surface of their plasma membranes (Fig. 2.4 D). Quantitative PCR revealed that *SPARC*$^{-/-}$ pericytes express higher basal expression of the TGFβ1-induced SMAD response genes *PAI-1*, *CTGF*, and *BIGH3* (Fig. 2.4 B). As increased TGFβ1 activity may arise from enhanced sensitivity to exogenous TGFβ1 present in the culture medium, we used shRNA knockdown of TGFβ1 to determine the contribution of endogenous TGFβ1 to this effect. Using phosphorylated SMAD2 (serines 465/467) as a readout of TGFβ1 activity, we found that knockdown of TGFβ1 was sufficient to reduce activity in *SPARC*$^{-/-}$ but not *SPARC*$^{+/+}$ pericytes (Fig. 2.4 C). Furthermore, *SPARC*$^{-/-}$ pericytes exhibited almost 2-fold higher levels of basal phospho-SMAD2, consistent with the observed increases in basal transcription (Fig. 2.4, C and B). We next asked whether endogenous TGFβ1 was responsible for the impaired migration of *SPARC*$^{-/-}$ pericytes using our transwell assay. Transfection with TGFβ1 shRNA but not control resulted in enhanced migration of *SPARC*$^{-/-}$ pericytes (Fig. 2.4 E). In the absence of SPARC, aberrant activity of TGFβ1 results in decreased pericyte migration.

SPARC interacts with the TGFβ accessory receptor endoglin

SPARC is a secreted glycoprotein; therefore, we hypothesized that control of TGFβ1 activity may be mediated through interactions with TGFβ1 receptors. Indeed,

SPARC has been shown to interact with a soluble form of TβRII but only in the presence of recombinant TGFβ1 (Francki et al., 2004). TGFβ1 binds sequentially to three receptors: active TGFβ1 first binds to TβRII, which then recruits a type I receptor. The TGFβ1/receptor complex can then recruit the type I membrane glycoprotein endoglin. We immunoprecipitated each of these receptors from 10T1/2 cells and found that SPARC specifically co-precipitated with endoglin (Fig. 2.5 B). We confirmed this interaction using solid phase binding assays where either the extracellular domain of endoglin or recombinant SPARC was used in the solid phase, and increasing concentrations of SPARC or endoglin were added in the aqueous phase (Fig. 2.5 C). Indirect immunofluorescent staining of primary *SPARC*$^{+/+}$ pericytes revealed that SPARC and endoglin associate in distinct punctate structures (Fig. 2.5 A). The interaction of SPARC with the TGFβ1 accessory receptor endoglin links SPARC with TGFβ1 function.

Endoglin localizes to focal complexes in SPARC deficient pericytes and blunts migration

The function of endoglin in TGFβ1 signaling is unclear; however, endoglin has been shown to modulate SMAD phosphorylation as well as control cell adhesion and migration, presumably through regulating the composition of focal adhesion complexes (Conley et al., 2004; Gougos et al., 1992; Koleva et al., 2006; Lee et al., 2008). Therefore, we explored whether endoglin exhibited differences in localization and activity in SPARC-deficient pericytes. We first assessed whether we could detect

endoglin associated with focal complexes of spreading *SPARC*$^{+/+}$ or *SPARC*$^{-/-}$ pericytes. We allowed primary cells to spread on fibronectin for one hour after which focal complexes and endoglin were visualized using anti-vinculin and anti-endoglin antibodies and indirect immunofluorescent staining. Vinculin-rich focal complexes were observed in spreading *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes (Fig. 2.6 A). Endoglin localized exclusively to distinct punctate structures in *SPARC*$^{+/+}$ pericytes and was not detected in focal complexes; however, the receptor co-localized with vinculin-rich focal complexes in *KO* pericytes (Fig. 2.6 A, insets and magnified panels). To determine whether endoglin aberrantly associates with the focal adhesion machinery in the absence of focal complexes, we immunoprecipitated endoglin complexes from *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes in suspension or seeded onto either tissue culture plastic or fibronectin and looked for the presence of FAK, as FAK is incorporated into maturing focal complexes of adherent cells (Kornberg et al., 1992). We found that FAK was present in endoglin immune complexes from *SPARC*$^{+/+}$ but not *SPARC*$^{-/-}$ pericytes, and that this association required cell adhesion (Fig. 2.6 B). *SPARC*$^{-/-}$ pericytes exhibit a TGFβ1-dependent impairment of migration; therefore, we hypothesized that focal complex-associated endoglin had a negative effect on cell migration. To test this, we knocked down endoglin in 10T1/2 cells while targeting SPARC with either shRNA or a monoclonal antibody and assessed migration using our transwell assay. Knockdown of endoglin had no effect on 10T1/2 cells transfected with control shRNA or treated with control antibody (Fig. 2.6 D). However, knockdown of endoglin did increase migration of cells transfected with SPARC

shRNA or treated with anti-SPARC antibody (Fig. 2.6 D) As *SPARC*$^{-/-}$ pericytes also exhibit elevated basal SMAD2 phosphorylation, we next asked whether mislocalization of endoglin to focal complexes and its negative role in migration correlated with endoglin-mediated induction of SMAD2 phosphorylation. Indeed, shRNA-mediated knockdown of endoglin resulted in a decrease in SMAD2 phosphorylation in *SPARC*$^{-/-}$ but not *SPARC*$^{+/+}$ pericytes (Fig. 2.6 C).

αV integrins interact with endoglin and positively regulate SMAD2 phosphorylation in SPARC deficient pericytes

Endoglin alone does not directly activate TGFβ1-mediated process (Koleva et al., 2006; Lee et al., 2008). Endoglin's association with focal complexes and regulation of migration and SMAD2 phosphorylation in *SPARC*$^{-/-}$ but not *SPARC*$^{+/+}$ pericytes suggested to us that perhaps, in the absence of SPARC, endoglin was cooperating with factors that positively regulate these processes. αV integrins facilitate cell adhesion and migration, and can directly activate TGFβ1 (Delannet et al., 1994; Klemke et al., 1994; Liaw et al., 1995; Stefansson and Lawrence, 1996); therefore we sought to determine if endoglin cooperated with αV integrins to enhance TGFβ1 activity in *SPARC*$^{-/-}$ pericytes. We found that *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes express similar levels of αV integrins by reverse transcriptase PCR and FACS (Fig. 2.7 A). Furthermore, *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes express αV integrin-binding β subunits that have been implicated in mediating TGFβ1 activation (Annes et al., 2004; Ludbrook et al., 2003; Mu et al., 2002; Munger et al., 1998).

Importantly, we found that αV integrins localized to focal complexes and that an αV integrin-blocking antibody impaired migration of both *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes (Fig. 2.7 B). We next examined whether αV integrin was present in endoglin immune complexes immunoprecipitated from cells in suspension or seeded onto either tissue culture plastic or fibronectin. αV integrin was detected in *SPARC*$^{-/-}$ pericyte immune complexes regardless of whether cells were adherent or in suspension (Fig. 2.7 D). αV integrin was also detected in *SPARC*$^{+/+}$ pericyte immune complexes, albeit at much lower levels (Fig. 2.7 D). As the level of αV integrin present in *SPARC*$^{-/-}$ endoglin immune complexes was greater than that seen in *SPARC*$^{+/+}$ complexes, we thought that SPARC may limit αV integrin incorporation into endoglin immune complexes. To test this hypothesis, we assessed the effect of adding recombinant SPARC to *KO* pericytes. We found that SPARC was able to reduce the amount of endoglin-associated αV integrin while having no effect on total αV integrin levels (Fig. 2.7 E). Finally, we asked whether endoglin-associated αV integrins are required for the increased basal TGFβ1 activity observed in *SPARC*$^{-/-}$ pericytes. Treatment of cells with an αV integrin-blocking antibody reduced SMAD2 phosphorylation in *SPARC*$^{-/-}$ but not *SPARC*$^{+/+}$ pericytes, though the extent of inhibition was not as great as that seen in cells treated with SB431542 (Fig. 2.7 C). Taken together, these results show that SPARC blocks αV integrins from interacting with endoglin and facilitating TGFβ1 signaling.

Figure legends

Figure 2.1. **SPARC is expressed by pericytes in vivo**.

(A) NG2 is expressed by pericytes in adult mouse pancreas. MECA32 and anti-NG2 antibodies were used to stain adult mouse pancreas. Bound antibodies were detected using indirect immunofluorescence. NG2 expression is restricted to pericytes which are associated with the $MECA32^{+}$ microvasculature. Inset is magnified in right panels. Bar = 100 um. (B, C) SPARC is expressed by pericytes in normal and PDAC pancreata. Anti-SPARC and anti-NG2 antibodies were used to stain pancreata from normal and PDAC (*P48Cre*$^{+}$*:LSLKras*$^{G12D+}$*:INK4A*$^{lox/lox}$) mice. SPARC expression is observed in $NG2^{+}$ cells of both *SPARC*$^{+/+}$ (B) and PDAC (C) pancreata. Insets are magnified in right panels, bars = 100 um. (D-F) PDAC tumors exhibit more pericyte-free vessels in the absence of SPARC expression. MECA32 and anti-NG2 antibodies were used to visualize the tumor vasculature of moribund *SPARC*$^{+/+}$ (D) and *SPARC*$^{-/-}$ (E) PDAC mice. An example of a pericyte-free vessel is presented in the inset of E and magnified in right panels. Bars = 100 um. (F) Percentage of pericyte-free vessels, vessel number, and relative vessel area (vessel area/ DAPI area) were assessed in tumors from 6 *SPARC*$^{+/+}$ and 8 *SPARC*$^{-/-}$ PDAC tumors. Values presented are mean + SEM (at least 18 20X fields/tumor, * = $P < 0.005$) *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$. (G) Isolation of primary pericytes. Top two rows: *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes were separated from collagenase-digested pancreata using anti-NG2 immunomagnetic bead separation. Purified cells express pericyte markers NG2 and desmin. Note that a

small subpopulation of these cells express α–SMA. Bottom panels: primary pericytes induce bEnd.3 cord formation. bEnd.3 cells were plated onto matrigel-coated chamber slides alone or in the presence of *SPARC*$^{+/+}$ or *SPARC*$^{-/-}$ pericytes in triplicate wells. Cord formation was assessed after 17 hours. The number of cords, number of branchpoints and cord widths were quantitated from five 10X fields per well. Experiment shown is representative of three independent experiments. Values are presented are mean + SEM (* $P < 0.0001$). Bar = 200 um. *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$.

Figure 2.2. ***SPARC*$^{-/-}$ pericytes exhibit defects in migration**.

(A) *SPARC*$^{-/-}$ pericytes exhibit decreased filopodia during spreading. Serum deprived primary *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes were allowed to adhere to fibronectin for 60 minutes. Cells were then fixed and stained with anti-vinculin IgG and Texas Red-conjugated phalloidin and visualized using fluorescent microscopy. Filopodia length and number were quantified on at least 20 cells using NIS-Elements software. Boxed regions are magnified in bottom panels. Bar = 20 um. White asterisks indicate filopodia. Values are presented as mean + SEM (* $P < 0.001$). *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$. (B) *SPARC*$^{-/-}$ pericytes exhibit larger and fewer mature focal adhesions. Primary *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes were allowed to adhere to fibronectin for 17 hours. Cells were then stained as in (A). Focal adhesions were counted as vinculin^{+} foci at the tips of actin stress fibers. Focal adhesion number and area were quantified in at least 20 cells using NIS-Elements software. Boxed regions are magnified in

bottom panels. Bar = 20 um. Values are presented as mean + SEM (* P < 0.005). *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$. (C) Primary *SPARC*$^{-/-}$ pericytes exhibit impaired migration. Serum deprived *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes were used in a modified Boyden chamber transwell assay in which membrane undersides were coated with fibronectin. 70,000 cells were seeded per transwell in the indicated conditions in triplicate and allowed to migrate for 6 hours. Cells that migrated to the membrane underside were manually counted in 20X fields covering the entire membrane. Data is presented as mean + SEM (* P < 0.02). *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$.

Figure 2.3. **TGFβ limits pericyte migration in the absence of SPARC**.

(A-C) Primary *SPARC*$^{-/-}$ but not *SPARC*$^{+/+}$ pericytes migrate in response to blocking TGFβ activity. (A) Neutralizing anti-TGFβ IgG enhances *SPARC*$^{-/-}$ pericyte migration in a transwell assay. 7,000 serum deprived primary *SPARC*$^{+/+}$ or *SPARC*$^{-/-}$ pericytes were seeded in triplicate into transwell inserts in which membrane undersides were coated with fibronectin as in Fig. 2. Cells were allowed to migrate towards fibronectin in the presence of no stimulation (NS), 10 nM PDGFB, 25 ng/ml anti-TGFβ IgG (α-TGFβ IgG), or 50 ng/ml VEGF-A for 6 hours. Cells that migrated to the membrane underside were manually counted in 20X fields covering the entire membrane. Experiment shown is representative of two independent experiments. Values are presented as mean + SEM (* P <0.05). *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$. (B) Inhibition of ALK5 enhances *SPARC*$^{-/-}$ pericyte migration in a transwell assay. Primary pericytes were allowed to migrate towards fibronectin in triplicate as in (A) in the presence of

25 ng/ml α-TGFβ or control IgG, 10 uM ALK5 inhibitor (SB431542), or ALK5 inhibitor vehicle alone (vehicle). Values are presented as mean + SEM (* P <0.05). *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$. (C) TGFβ neutralization and ALK5 inhibition increase migration of *SPARC*$^{-/-}$ pericytes in a scratch assay. Primary pericytes were seeded at confluency in 96 well-plate wells coated with fibronectin in quadruplet. A mechanically denuded area was introduced and cells were allowed to migrate overnight either alone, in the presence of 25 ng/ml α-TGFβ IgG, or 10 uM ALK5 inhibitor (SB431542). Denuded area was measured before and after migration using Elements software and normalized to cell viability as measured by the Cell Titer Blu assay (Promega). 100% migration was taken as the average of the resulting values (area/viability) from the 'cells alone' wells. Values are presented as mean + SEM (* P <0.05). *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$. (D) Knockdown of SPARC in 10T1/2 cells impairs migration in a TGFβ-dependent manner. 10T1/2 cells were transfected with SPARC or control shRNA for 48 hours then seeded at 5,000 cells into fibronectin-coated transwell inserts in triplicate as in (A) and (B). Western blot to confirm knockdown is presented in left panels. Cells were allowed to migrate in the presence of 25 ng/ml α-TGFβ IgG. Cells that migrated to the membrane underside were manually counted in 20X fields covering the entire membrane. Values are presented as mean + SEM (* P <0.05). (E) Anti-SPARC IgG reduces 10T1/2 cell migration in a TGFβ-dependent manner. 10T1/2 cells were allowed to migrate towards fibronectin as in D in the presence of 25 ng/ml control IgG, 25 ng/ml anti-SPARC IgG clone 303 (α-SPARC IgG), 25 ng/ml α-TGFβ IgG + 25 ng/ml α-SPARC IgG, or 25 ng/ml α-TGFβ

IgG + 25 ng/ml control IgG for 6 hours. Cells that migrated to the membrane underside were manually counted in 20X fields covering the entire membrane. Values are presented as mean + SEM (* P <0.05).

Figure 2.4. ***SPARC*$^{-/-}$ pericytes exhibit increased basal TGFβ activity.**

(A) Primary *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes express similar levels of TGFβ1 protein. Left panel: whole cell lysates from *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes contain similar levels of TGFβ1. 150,000 cells were plated in triplicate into fibronectin coated 6-well plates and cultured in the presence of low serum media (0.75 % serum) for up to 96 hours. Whole cell lysates were prepared every 24 hours in lysis buffer and stored at -20^{o} C until assay was completed. TGFβ levels were assessed using a capture ELISA kit (Promega). Conditioned media from every timepoint was also harvested and probed for active TGFβ (left panel). Pg/ml TGFβ1was calculated by interpolating experimental ELISA measurements from a linear curve generated from a TGFβ1 standard using Prizm software. Experiments shown are representative of two independent experiments. Values are presented as mean + SEM (* P < 0.002). *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$. (B) Primary *SPARC*$^{-/-}$ pericytes exhibit increased TGFβ-induced transcription. Left panels: reverse transcription PCR of primary pericytes grown on plastic or fibronectin. Cells were seeded at approximately 80% confluency and incubated overnight, followed by RNA extraction and cDNA synthesis. Note the more intense *PAI-1* band from KO pericyte cDNA from cells plated on both plastic and fibronectin. Right panels: TGFβ1 induces transcription in

both *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes. Serum deprived primary pericytes were treated with the indicated concentrations of recombinant TGFβ1 for 17 hours in triplicate. cDNA was then prepared and used for SYBR green qPCR using primers specific for TGFβ1-response genes *PAI-1*, *CTGF*, and *BIGH3.* qPCR reactions were performed in triplicate. Fold change in expression was calculated using delta delta Ct method where *WT* at 0 pg/ml TGFβ1 was the reference sample and *GAPDH* was the reference gene. *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$. (C) Knockdown of TGFβ1 reduces basal SMAD2 phosphorylation in *SPARC*$^{-/-}$ pericytes. Primary pericytes were transfected with TGFβ1 or control shRNA for 48 hours in the presence of 20 ug/ml bovine serum albumin (BSA) or recombinant SPARC (rSPARC) as indicated. Cell lysates were probed for phosphorylated SMAD2 (serine 465/467, pSMAD2) and total SMAD2 (tSMAD2) by western blot (right panel). pSMAD2 bands were normalized to tSMAD2 bands using ImageJ software. Knockdown was validated using SYBR green qPCR where fold change was calculated using untreated *SPARC*$^{+/+}$ pericyte cDNA as the reference sample and *GAPDH* as the reference gene (left panel). Experiment shown is representative of two independent experiments. *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$. (D) *SPARC*$^{-/-}$ pericytes retain more surface TGFβ1than *SPARC*$^{+/+}$ counterparts. Primary pericytes were grown to confluency. Surface proteins were labeled with a cell-impermeable and cleavable biotinylation reagent (Sulfo-NHS-SS-biotin) and purified using Neutravidin affinity chromatography. Surface proteins were subjected to SDS-PAGE and were probed for the indicated proteins by western blot. *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$. (E) Knockdown of TGFβ1 enhances migration of

primary *SPARC*$^{-/-}$ pericytes. Pericytes were transfected with the indicated shRNA for 48 hours then used in a transwell migration assay and allowed to migrate towards fibronectin in triplicate as in Fig. 3. Cells that migrated to the membrane underside were manually counted in 20X fields covering the entire membrane. Experiment shown is representative of two independent experiments. Values are presented as mean + SEM (* P <0.05) *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$.

Figure 2.5. **SPARC interacts with TGFβ1 accessory receptor endoglin.**

(A) SPARC and endoglin colocalize in primary *SPARC*$^{+/+}$ pericytes. Pericytes were allowed to adhere to silane coated slides overnight then stained with anti-SPARC IgG, anti-endoglin IgG. Bound antibodies were detected using indirect immunofluorescence. DAPI was used to visualize nuclei. Bar = 20 um. (B) SPARC coprecipitates with endoglin immune complexes from 10T1/2 cells. 10T1/2 cell lysates were incubated anti-ALK5, anti-endoglin, anti-SPARC, or anti-TβRII (RII) IgGs. IgG-bound complexes were precipitated with protein A/G agarose beads. The indicated immune complexes were subjected to SDS-PAGE and probed for the indicated proteins by western blot. Asterisks indicate the target protein. Experiments shown are representative of several independent experiments. IgG heavy chain appears as the 50 kilodalton band in each western blot. kDa = kilodalton, IP = immunoprecipitation, WB = western blot. (C) SPARC interacts with endoglin in solid phase binding assays. 96-well plates were coated with either 5 ug/ml endoglin (left panel) or 5 ug/ml SPARC (right panel) or control serum. Soluble SPARC or endoglin

was added to the indicated plate in triplicate at increasing concentrations. Wells were then washed and bound protein was detected using anti-SPARC or anti-endoglin IgG. These antibodies were detected with horse radish peroxidase-conjugated secondary antibodies, using TMB as the developing agent. Experiment shown is representative of three independent experiments. Binding is expressed as average absorbance +/- SEM.

Figure 2.6. **Endoglin associates with focal complexes in *SPARC*$^{-/-}$ pericytes.**

(A) Endoglin colocalizes with vinculin in *SPARC*$^{-/-}$ pericytes. Primary *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes were seeded onto fibronectin coated slides and allowed to spread for 60 minutes. Cells were then fixed and stained with anti-vinculin and anti-endoglin IgG. Bound antibodies were visualized using indirect immunofluorescence. Boxed regions are magnified in adjacent panels. Bar = 20 um. *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$. (B) FAK coprecipitates with endoglin immune complexes from *SPARC*$^{-/-}$ pericytes. Endoglin was immunoprecipitated from primary pericyte lysates harvested from cells in suspension (S), or cells adhered to either plastic (P) or fibronectin (F). Immune complexes were then subjected to SDS-PAGE and probed for FAK and endoglin by western blot. FAK was also detected in whole cell lysates (WCL). Experiment shown is representative of two independent experiments. *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$. (C) Knockdown of endoglin reduces SMAD2 phosphorylation in *SPARC*$^{-/-}$ pericytes. Cell lysates were prepared from primary pericytes transfected with endoglin or control shRNA for 48 hours. Lysates were subjected to SDS-PAGE and phosphorylated

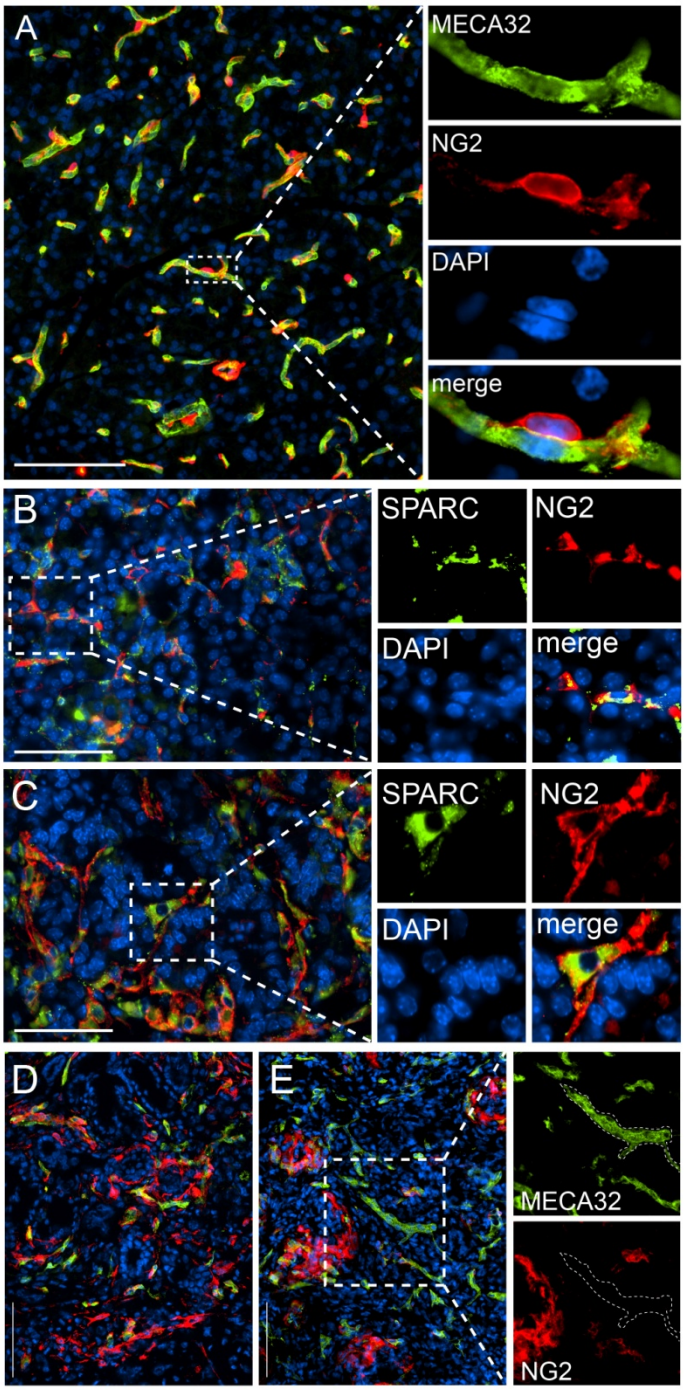
SMAD2 (serine 465/467, pSMAD2) and total SMAD2 (tSMAD2) were detected by western blot. pSMAD2 levels were normalized to tSMAD2 using ImageJ software. Experiment shown is representative of two independent experiments. *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$. (D) Knockdown of endoglin reverses the effect of targeting SPARC on 10T1/2 cell migration. 10T1/2 cells were transfected with the indicated shRNA for 48 hours then used in a transwell assay at 7,000 cells per transwell in triplicate. Cells were allowed to migrate towards fibronectin in the presence or absence of 25 ng/ml anti-SPARC or control IgG as indicated. Cells that migrated to the membrane underside were manually counted in 20X fields covering the entire membrane. Experiment shown is representative of three independent experiments. Values are presented as mean + SEM (* $P < 0.05$).

Figure 2.7. **αV integrin interacts with endoglin and mediates TGFβ activity in primary *SPARC*$^{-/-}$ pericytes.**

(A) Integrin expression profile of primary pericytes. cDNA was prepared from cells cultured overnight on fibronectin-coated dishes in 0.75% serum and used for reverse transcription PCR detection of SPARC, αV integrin (*itgav*), β1 integrin (*itgb1*), β3 integrin (*itgb3*), β6 integrin (*itgb6*), and RPS6 (*rps6*). Right panels: primary *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes express αV integrins at their surfaces. 500,000 were seeded onto fibronectin-coated dishes and allowed to adhere overnight in 0.75% serum media. Cells were harvested and prepared for FACS analysis using anti-αV integrin IgG (RMV-7) at 20 ug/ml and Cy3-conjugated donkey anti-rat IgG secondary.

Control cells were stained with Cy3-conjugated donkey anti-rat IgG alone. *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$. (B) αV integrins regulate migration of primary pericytes. Serum deprived primary pericytes were seeded in transwell migration inserts in triplicate at 7,000 cells per transwell and allowed to migrate towards fibronectin for 6 hours. Cells were allowed to migrate alone, in the presence of 20 ug/ml RMV-7, or in the presence of 20 ug/ml control IgG. Cells that migrated to the membrane underside were manually counted in 20X fields covering the entire membrane. Experiment shown is representative of two independent experiments. Values are presented as mean + SEM (* P <0.05). *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$. (C) αV integrins associate with endoglin in *SPARC*$^{-/-}$ pericytes. Endoglin was immunoprecipitated from primary cell lysates harvested from cells in suspension (S), or cells adhered to either plastic (P) or fibronectin (F). Immune complexes were then subjected to SDS-PAGE and probed for αV integrin by western blot.WCL = whole cell lysates, IP = immunoprecipitation, *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$. Experiment shown is representative of two independent experiments. (D) αV integrins regulate TGFβ activity in primary *SPARC*$^{-/-}$ pericytes. Primary *SPARC*$^{+/+}$ or *SPARC*$^{-/-}$ pericytes were incubated overnight in 1.5% serum in the presence of 10 uM ALK5 inhibitor (SB431542), 20 ug/ml RMV-7, 20 ug/ml control IgG, or vehicle alone as indicated. Whole cell lysates were then prepared and subjected to SDS-PAGE. Phospho-SMAD2 (serine 465/467, pSMAD2) and total SMAD2 (tSMAD2) were probed for by western blot. pSMAD2 levels were normalized to tSMAD2 using ImageJ software. Experiments shown are representative of three independent experiments. *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$. (E) SPARC

blocks αV integrin/endoglin interaction. *SPARC*$^{-/-}$ pericytes were incubated with either bovine serum albumin (BSA) or recombinant SPARC (rSPARC) at the indicated concentrations for 6 hours. Endoglin was immunoprecipitated from cell lysates, and immune complexes were subjected to SDS-PAGE. αV integrin and SPARC were probed for by western blot. Coprecipitating αV integrin was normalized to αV integrin in whole cell lysates using ImageJ software. WCL = whole cell lysates, IP = immunoprecipitation *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$. Experiment shown is representative of two independent experiments. (F) Model of SPARC-mediated control of pericyte migration. SPARC blocks activity of TGFβ1 from impairing pericyte migration and inducing SMAD2 phosphorylation. The inhibitory activity of TGFβ1 on pericyte migration is mediated by endoglin and αV integrins.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MECA32
NG2
DAPI
merge
B
SPARC
NG2
DAPI
merge
C
SPARC
NG2
DAPI
merge
D
E
MECA32
NG2

FIGURE 2.1, A-E

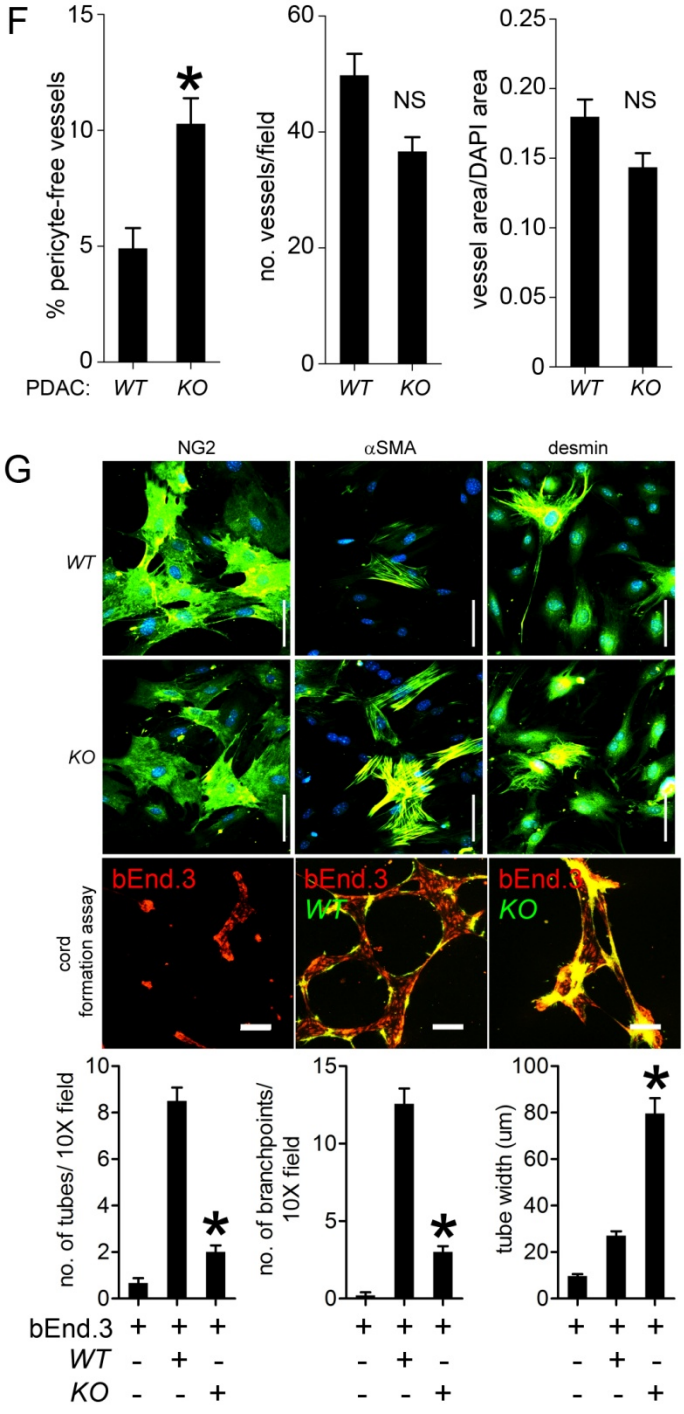
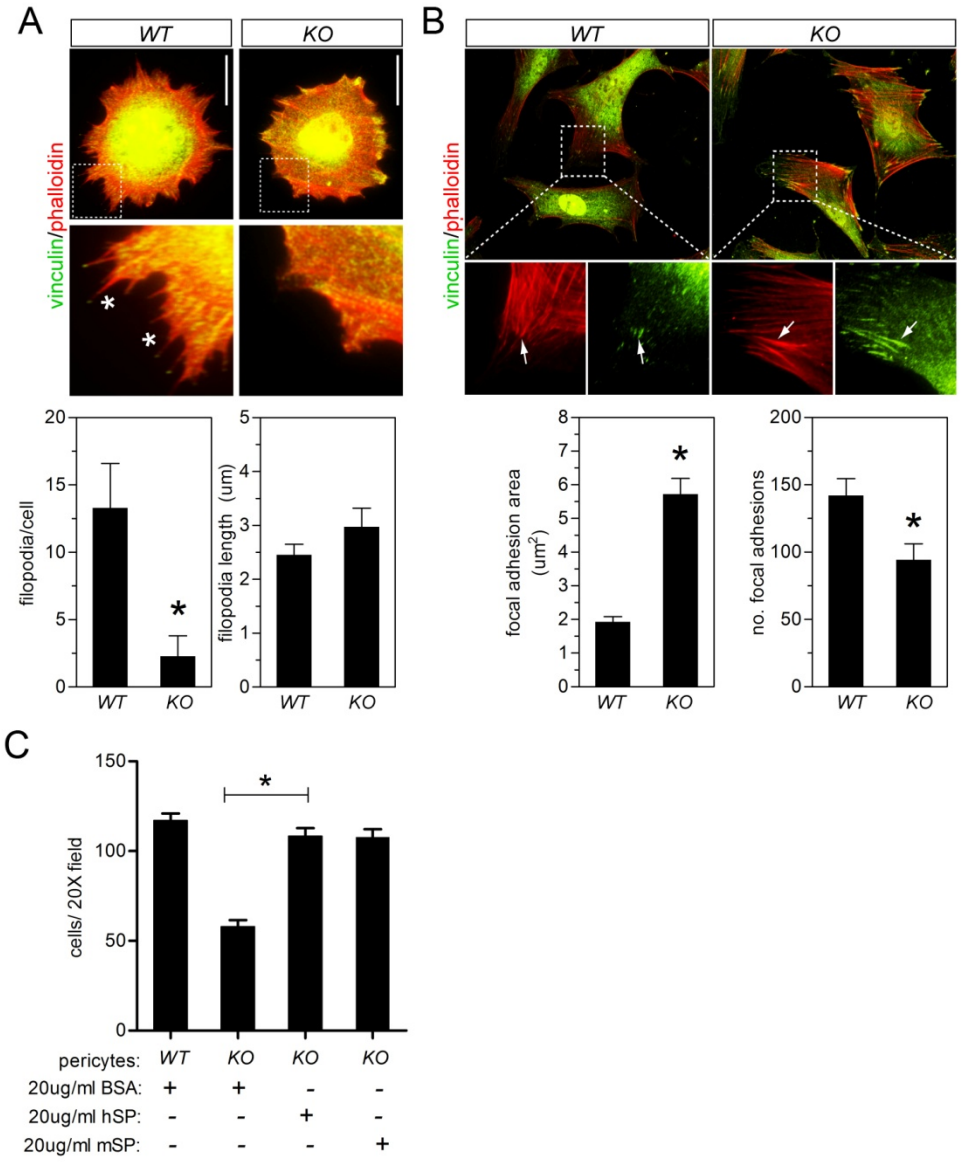


FIGURE 2.1, F AND G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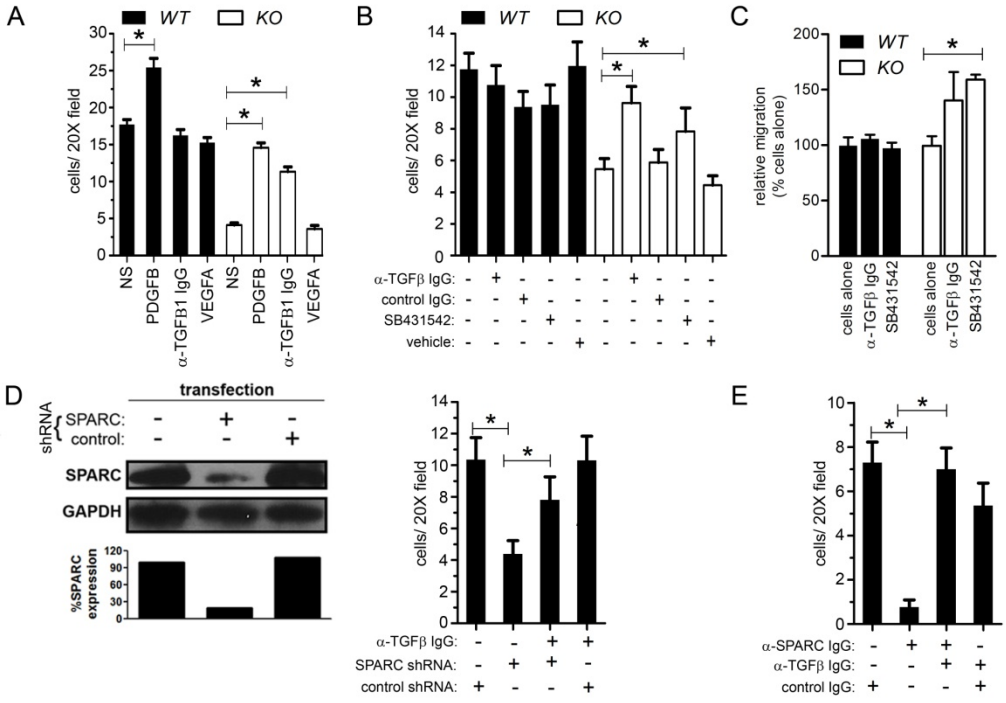
WT
KO
vinculin/phalloidin
filopodia/cell
filopodia length (um)
B
focal adhesion area (um²)
no. focal adhesions
C
cells/ 20X field
pericytes: WT KO KO KO
20ug/ml BSA: + + - -
20ug/ml hSP: - - + -
20ug/ml mSP: - - - +

FIGURE 2.2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WT
KO
cells/ 20X field
NS
PDGFB
α-TGFβ1 IgG
VEGFA
B
α-TGFβ IgG:
control IgG:
SB431542:
vehicle:
C
relative migration (% cells alone)
cells alone
α-TGFβ IgG
SB431542
D
transfection
shRNA
SPARC:
control:
SPARC
GAPDH
%SPARC expression
SPARC shRNA:
control shRNA:
E
α-SPARC IgG:
α-TGFβ IgG:
control IgG:

FIGURE 2.3

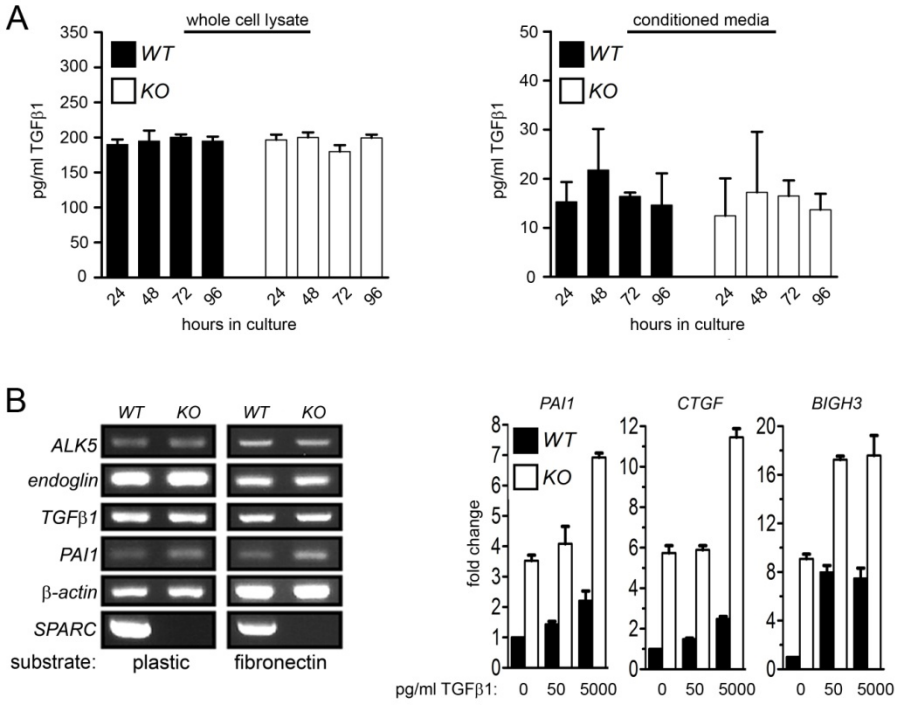


FIGURE 2.4, A AND B

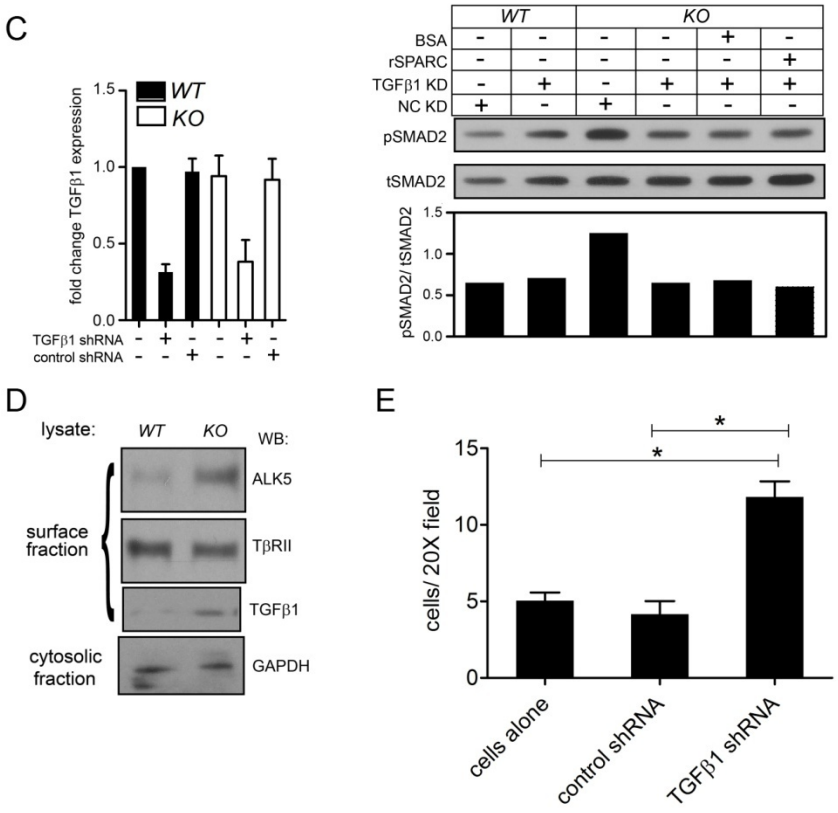
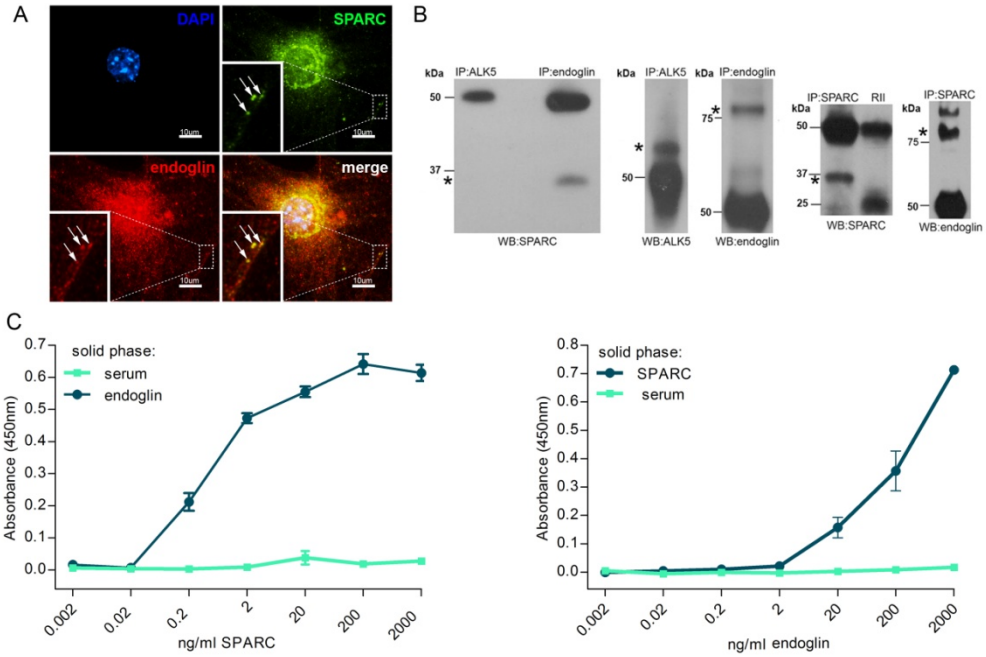

C
WT
KO
fold change TGFβ1 expression
1.5
1.0
0.5
0.0
TGFβ1 shRNA - + - - + -
control shRNA - - + - - +
WT
KO
BSA - - - - + -
rSPARC - - - - - +
TGFβ1 KD - + - + + +
NC KD + - + - - -
pSMAD2
tSMAD2
pSMAD2/ tSMAD2
1.5
1.0
0.5
0.0
D
lysate: WT KO WB:
ALK5
surface fraction
TβRII
TGFβ1
cytosolic fraction
GAPDH
E
cells/ 20X field
15
10
5
0
*
*
cells alone
control shRNA
TGFβ1 shRNA

FIGURE 2.4, C – E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DAPI
SPARC
endoglin
merge
10um
B
kDa
IP:ALK5
IP:endoglin
50
37
WB:SPARC
WB:ALK5
WB:endoglin
IP:SPARC
RII
25
75
C
solid phase:
serum
endoglin
SPARC
Absorbance (450nm)
ng/ml SPARC
ng/ml endoglin
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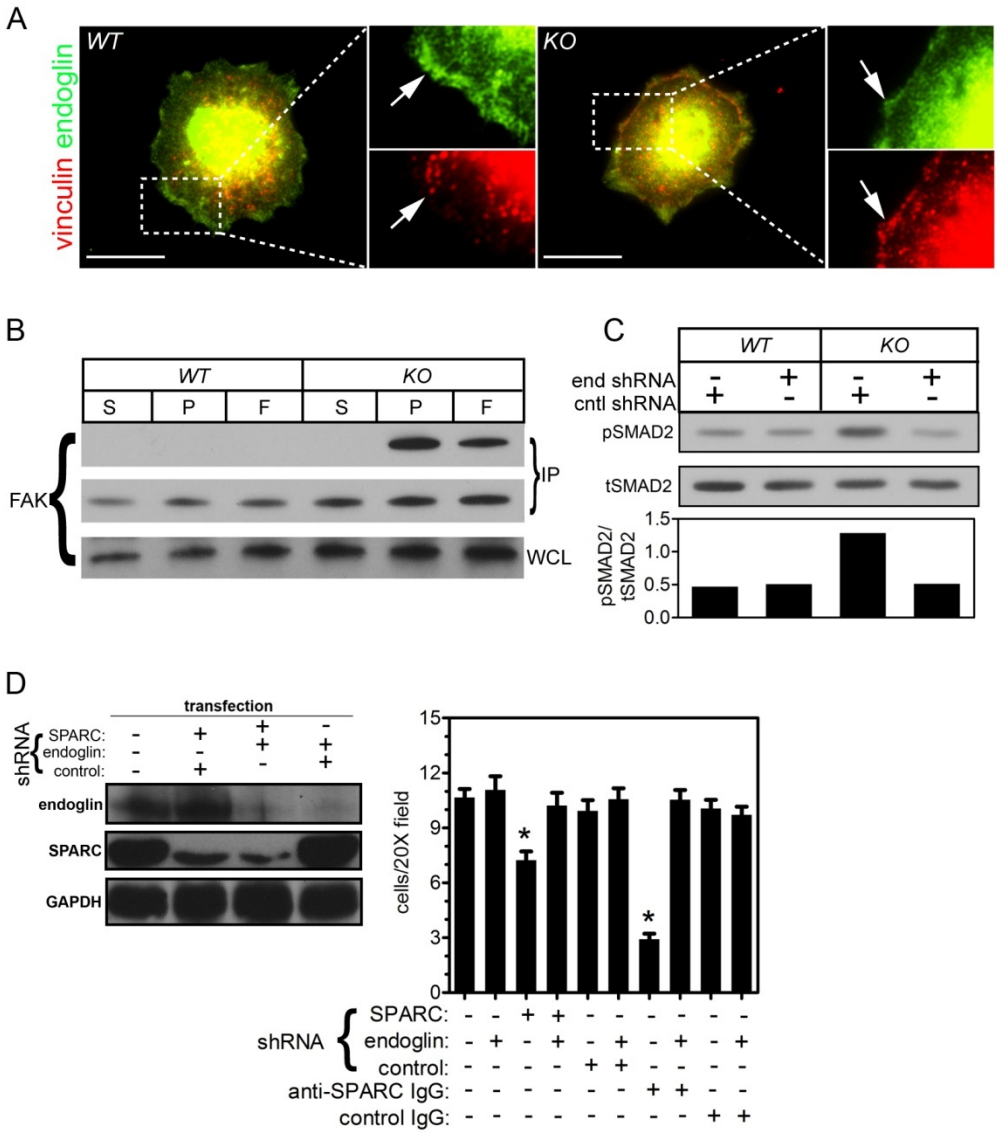
0.02
0.2
2
20
200
2000

FIGURE 2.5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WT
KO
vinculin endoglin
B
WT
KO
S
P
F
S
P
F
FAK
IP
WCL
C
WT
KO
end shRNA
cntl shRNA
pSMAD2
tSMAD2
pSMAD2/
tSMAD2
1.5
1.0
0.5
0.0
D
transfection
shRNA
SPARC:
endoglin:
control:
endoglin
SPARC
GAPDH
cells/20X field
15
12
9
6
3
0
shRNA
SPARC:
endoglin:
control:
anti-SPARC IgG:
control IgG:

FIGURE 2.6

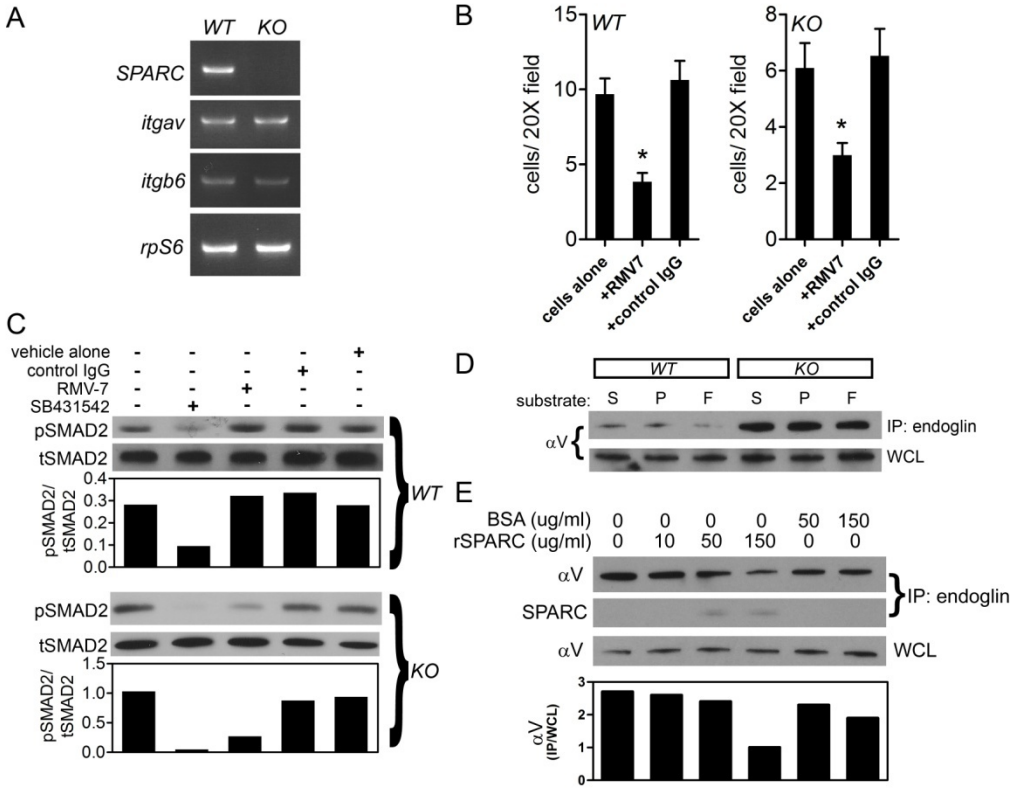

A
WT
KO
SPARC
itgav
itgb6
rpS6
B
WT
KO
cells/ 20X field
cells alone
+RMV7
+control IgG
C
vehicle alone
control IgG
RMV-7
SB431542
pSMAD2
tSMAD2
pSMAD2/ tSMAD2
WT
KO
D
substrate: S P F S P F
αV
IP: endoglin
WCL
E
BSA (ug/ml) 0 0 0 0 50 150
rSPARC (ug/ml) 0 10 50 150 0 0
αV
SPARC
IP: endoglin
WCL
αV (IP/WCL)

FIGURE 2.7

CHAPTER 3

Discussion and future directions

Regulation of TGFβ1activation by SPARC in pericytes

The activation of TGFβ1 on pericytes occurs as pericytes contact nascent endothelial tubes during angiogenesis. The spatial and temporal regulation of this activation is crucial, as aberrant activation results in deficient pericyte recruitment and defective vascular function. Using α–SMA as a pericyte marker, previous studies found that orthotopic tumors grown in *SPARC*$^{-/-}$ mice exhibited a decrease in the number of pericyte associated blood vessels, suggesting that SPARC regulates pericyte behavior (Arnold et al., 2010; Puolakkainen et al., 2004). This phenotype could be due to differences in the tumor microenvironment resulting from the presence or absence of SPARC or to some pericyte autonomous effects(s) of SPARC expression; however, these studies did not determine whether SPARC was expressed by pericytes or whether SPARC expression could modify pericyte behavior. In the current study, I identify SPARC as a novel regulator of pericyte behavior. Pericytes were found to express relatively high levels of SPARC in normal adult mouse pancreas as well as in PDAC lesions undergoing angiogenesis, suggesting that SPARC participates in regulating the behavior of these cells in vivo (Fig. 2.1, A-C). Interestingly, SPARC expression was most obvious in the NG2^{+} pericyte populations, suggesting that pericytes provide the majority of secreted SPARC from normal quiescent pancreas and PDAC. To determine the function of SPARC expression in pericytes, I crossed our PDAC mouse line into *SPARC*$^{-/-}$ mice. Surprisingly, SPARC deficiency in PDAC resulted in 2-fold increase in pericyte-free vessels, without affecting blood vessel number, demonstrating that the observed SPARC expression in

pericytes is required for normal pericyte behavior and their recruitment to blood vessels, but is not required for blood tumor blood vessel formation (Fig. 2.1, D-F). To better understand the function of SPARC in dictating pericyte behavior, I isolated primary pericytes from adult pancreata from *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ mice using immunomagnetic bead separation with anti-NG2 antibodies. I hypothesized that since there was a deficiency in the number of pericyte-associated vessels in *SPARC*$^{-/-}$ PDAC tumors, *SPARC*$^{-/-}$ pericytes would be exhibit abnormal behavior in endothelial cell cocultures. Indeed, I found that primary pericytes isolated from the pancreata of *SPARC*$^{-/-}$ mice were less able to induce bEnd.3 cord formation (Fig. 2.1, G). Furthermore, it appeared as though *SPARC*$^{-/-}$ pericytes aggregated into cell clusters, whereas *SPARC*$^{+/+}$ pericytes appeared to have migrated along the lengths of the bEnd.3 cords so that they were sparsely but evenly distributed throughout. Both *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes were able to associate with bEnd.3 cells. As these defects in pericyte behavior were possibly due to defects in cell migration, I examined filopodia and focal adhesion formation on fibronectin-coated surfaces and found that spreading *SPARC*$^{-/-}$ pericytes exhibited decreased filopodia and that fully spread *SPARC*$^{-/-}$ pericytes had larger but fewer focal adhesions, consistent with mobility defects (Fig. 2.2, A and B). To confirm that differences in filopodia and focal adhesion formation corresponded with defective cell mobility, I assayed cell migration directly using a transwell assay in which the underside of the transwell membrane was coated with fibronectin (Fig. 2.2 C). I found that *SPARC*$^{-/-}$ pericytes were less able to migrate in this assay, and that incubation of *SPARC*$^{-/-}$ cells with recombinant SPARC

protein rescued their impaired migration, demonstrating for the first time that SPARC is a positive regulator of pericyte migration.

TGFβ1 is a pleotrophic cytokine that negatively regulates pericyte migration in vivo, and SPARC has been demonstrated to regulate the activity of this cytokine in a variety of experimental conditions. However, previous studies have yielded conflicting conclusions on the functional relationship between SPARC and TGFβ1. Studies with mouse mesangial cells found that SPARC positively regulates TGFβ1 activity and enhances SMAD2 phosphorylation, presumably through interactions with TβRII and TGFβ1, while experiments with epithelial and endothelial cells have demonstrated that SPARC can enhance SMAD1 and SMAD2 phosphorylation in a TGFβ1-dependent manner (Francki et al., 1999; Francki et al., 2004; Schiemann et al., 2003). In contrast to these results, a recent study found that SPARC blocked TGFβ1 activity in fibroblasts in vivo and in vitro (Chlenski et al., 2007). The latter supports a model where SPARC functions to block TGFβ1 signaling in pericytes. I reasoned that if SPARC blocked TGFβ1 activity, the decrease in *SPARC*$^{-/-}$ pericyte migration may be due to elevated TGFβ1 activity. Therefore, I hypothesized that in the absence of SPARC, pericytes would exhibit a TGFβ1 induced impairment of cell migration. Comparison of pericyte migration in response to PDGFB, VEGF-A, and the pan-TGFβ neutralizing antibody 1D11 revealed that *SPARC*$^{-/-}$ but not *SPARC*$^{+/+}$ pericytes could migrate more effectively in the absence of TGFβ (Fig. 2.3, A). To determine whether TGFβ1 signaling was playing a role in this effect, I compared migration of

pericytes treated with 1D11 to pericytes treated with the TGFβ type I receptor ALK5 kinase inhibitor SB431542 (Fig. 2.3 B and C). Both inhibitors enhanced migration of *SPARC*$^{-/-}$ pericytes to the same extent. These results imply that SPARC blocks TGFβ1 activity from impairing pericyte migration. To determine if this effect was specifically the result of SPARC expression, I assessed whether knockdown of SPARC in the pericyte-like 10T1/2 cell line could recapitulate the behavior of *SPARC*$^{-/-}$ pericytes. The results of these experiments were in line with what I observed with primary pericyte: knockdown of SPARC resulted in TGFβ-mediated impairment of migration (Fig. 2.3, D). The "promigratory", anti-TGFβ1 effect of SPARC was likely mediated by secreted SPARC as neutralization of SPARC with the anti-SPARC monoclonal antibody 303 had a similar, yet more robust effect on 10T1/2 cell migration (Fig. 2.3 E).

I hypothesized that *SPARC*$^{-/-}$ pericytes were susceptible to the effects of TGFβ because they expressed more TGFβ compared to *SPARC*$^{+/+}$ pericytes, making the extracellular concentration of ligand higher. Therefore, I assessed TGFβ1 expression in these cells. I found that levels of both cell-associated and secreted total TGFβ1 were similar in *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes (Fig. 2.4 A). Furthermore, I found no changes in expression levels of TGFβ1 reverse transcription PCR (Fig. 2.4 B). I then probed the surfaces of primary pericytes for the presence of active TGFβ1, ALK5, and TβRII and found that *SPARC*$^{-/-}$ pericyte surfaces had higher levels of both ALK5 and active TGFβ1 (Fig. 2.4 D). Taken together, these results suggest that neither TGFβ1

expression nor secretion depend on SPARC expression, rather, active TGFβ1 becomes associated preferentially with *SPARC*$^{-/-}$ pericyte surfaces. This may be due to enhanced processing of latent TGFβ1 at the surfaces of *SPARC*$^{-/-}$ pericytes; however, that I was not able to detect measurable differences in secreted TGFβ1 protein between *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes, it may also be that there is decreased turnover of active TGFβ1 from the *SPARC*$^{-/-}$ surfaces. This scenario would be in line with the increase in surface-associated ALK5 in *SPARC*$^{-/-}$ cells, which suggests that TGFβ1 is maintained at the surface while bound to a subset of its receptors.

SPARCs capacity to block TGFβ1 activity in pericytes supports previous work showing that SPARC could inhibit TGFβ1-mediated SMAD phosphorylation and α–SMA expression in fibroblasts (Chlenski et al., 2007). Since sensitivity of pericyte migration to TGFβ activity was dependent on the presence of SPARC, I hypothesized that *SPARC*$^{-/-}$ pericytes exhibit increased basal TGFβ-induced activity. I found that *SPARC*$^{-/-}$ pericytes expressed higher basal expression of *CTGF*, *PAI-1*, and *BIGH3*, three SMAD-dependent TGFβ1-induced genes (Fig. 2.4 B). Furthermore, these cells exhibited increased basal SMAD2 phosphorylation (Fig. 2.4 C). Since *SPARC*$^{-/-}$ pericytes exhibited elevated basal TGFβ1 signaling and the ability to block TGFβ1-induced impairment of *SPARC*$^{-/-}$ pericyte migration with a neutralizing antibody did not require addition of exogenous TGFβ1, and, I hypothesized that endogenously expressed TGFβ1 was mediating enhanced TGFβ1 activity in *SPARC*$^{-/-}$ pericytes. Knockdown of TGFβ1 reduced SMAD2 phosphorylation to levels observed

in *SPARC*$^{+/+}$ pericytes, and enhanced migration of *SPARC*$^{-/-}$ pericytes (Fig. 2.4, C and E). These results demonstrate that endogenous SPARC blocks TGFβ1 from impairing pericyte migration without increasing TGFβ1 expression. This suggests that SPARC is able to decrease TGFβ1 activity immediately upon contact with the pericyte membrane surface, acting to help direct the pericyte during angiogenesis.

Identification of SPARC expression by pericytes in both resting and angiogenic vasculature suggest that its activity is not regulated by expression alone. SPARCs function as an inhibitor of TGFβ1 activation and positive regulator of pericyte migration must be spatially and temporally controlled during angiogenesis, as pericytes must eventually activate TGFβ1 signaling at the point of contact with a nascent vessel; constitutive expression of SPARC by pericytes would thusly interfere with vessel stabilization if expression was the only level of regulating its activity. One possibility is that SPARCs affect on TGFβ1 activity in pericytes is dependent on the protease plasmin. Plasmin has been shown to cleave SPARC, releasing SPARC-derived angiogenenic GHK-containing peptide (Iruela-Arispe et al., 1995). These peptides stimulate endothelial cell proliferation but their effect on pericytes has not been established. Using the chick chorioallantoic membrane assay for angiogenesis, it was found that SPARC expression spatially overlapped that of plasmin, and that plasmin levels were highest during the early phase of angiogenesis (Iruela-Arispe et al., 1995). This correlates with plasmin/SPARC-derived GHK-containing cleavage products inducing proangiogenic responses during the initial phases of angiogenesis. As the angiogenic response comes to completion, plasmin levels drop, and SPARC-

derived GHK-containing peptide levels diminish as does the effect of SPARC angiogenesis. Therefore, regulation of SPARCs affect on TGFβ1 activity in pericytes by plasmin seems a plausible mechanism by which SPARCs function could be spatially and temporally regulated.

SPARC$^{-/-}$ pericytes reveal a novel role for endoglin in regulating pericyte behavior

Endoglin has traditionally been used as a marker for activated endothelial cells. However, it was originally identified as an antigen that interacted with the monoclonal antibody 44G4, which was derived from mice immunized with human non-B, non-T, acute lymphocytic leukemia cells (HOON cells) (Quackenbush and Letarte, 1985). 44G4 was shown to bind to a single protein with a reduced molecular weight of approximately 95,000 and a non-reduced molecular weight of 125,000. This protein was found to be a glycosylated dimer expressed on the surfaces of acute myeloid leukemia cells, bone marrow cells, and cells from dissociated tonsils. Immunohistochemical staining of kidney sections with 44G4 revealed that the 44G4's antigen was expressed by interstitial and peritubular capillaries, as well as by the mesangium of glomeruli (Quackenbush et al., 1986). The vascular pattern of staining was also observed in histological tissue sections of lymph node, tonsil, thymus, spleen, liver, lung, and umbilical cord (Gougos and Letarte, 1988). 44G4 was then shown to be non-reactive with B-cells, granulocytes, monocytes, and resting or phytohemagglutinin-activated T cells, but reactive with human umbilical cord-derived

endothelial cell cultures. Human umbilical vein endothelial cells were then used to produce a phage cDNA library that yielded the primary structure of the endoglin gene product (Gougos and Letarte, 1990). As immunohistological staining with 44G4 releaved a vascular pattern of expression, and endothelial cells expressed high levels of this protein, endoglin was deemed an endothelial cell marker; however, careful examination of the vascular staining pattern in lymph nodes reveals that, in addition to the capillary endothelial cells, perivascular cells also express the 44G4 antigen.

Assessment of endoglin's primary structure revealed the protein was likely a type I integral membrane protein, having its NH_2 terminus exposed to the extracellular space (Gougos and Letarte, 1990). The predicted structure had a 561 amino acid extracellular domain, a 25 amino acid single pass transmembrane domain, and a 47 amino acid cytoplasmic tail. In addition, there was an RGD motif located in the predicted extracellular domain, suggesting that endoglin may regulate cell adhesion by interacting with RGD-binding integrins. This domain also contained several potential *N*-linked and *O*-linked glycosylation sites, which was consistent with observed molecular weight shifts after glycosidase treatment of purified endoglin protein.

Shortly after the identification of endoglin in endothelial cells and capillaries, the primary structure of betaglycan revealed these two proteins share significant identity and homology within their transmembrane and cytoplasmic domains (Lopez-Casillas et al., 1991). Betaglycan is a type III TGFβ accessory receptor which had already been described as a TGFβ1 receptor before the elucidation of its primary structure; this hinted at a role for endoglin in TGFβ1 signaling. Endoglin was then

found to interact with TGFβ1 on the surfaces of endothelial cells (Cheifetz et al., 1992; McAllister et al., 1994). Furthermore, unlike betaglycan, which binds to TGFβ1, TGFβ2, and TGFβ3, endoglin was found to associate exclusively with TGFβ1 and TGFβ3.

Shortly after its initial characterization, autosomal dominant mutations in the endoglin gene were found to be responsible for causing hereditary hemorrhagic telangiectasia type 1 (McAllister et al., 1994). Hereditary hemorrhagic telangiectasia is a systemic vascular disease characterized by various malformations in the vasculature, which are primarily caused by the absence of capillaries (Govani and Shovlin, 2009). This leads to excess microvessel pressure resulting in dysplasia and hemorrhaging. Later, studies of genetically modified mice revealed that endoglin contributes to blood vessel formation and stabilization. Endoglin promoter-driven beta-galactosidase expression revealed that endoglin was expressed by the developing vasculature (Arthur et al., 2000; Bourdeau et al., 1999). Homozygous endoglin null mice died in utero at E10- 10.5 (Bourdeau et al., 1999). Developmental analyses revealed that both embryonic and yolk sack vasculatures developed normally until embryonic day 9. Yolk sacks from E9-9.5 homozygous null mice were characterized by an abnormal vascular plexus having endothelial cell-lined vascular channels but lacking branching. The vascular channels Ire enlarged and leaked erythrocytes into the yolk sack cavities. Assessment of the embryonic vasculature revealed that E9 homozygous null mice exhibited extensive hemorrhaging with blood pools present in the peritoneal cavity. Analysis of E8 embryos revealed that vasculogenesis occurs

similarly in wildtype and homozygous null mice (Li et al., 1999). At E8.5, differentiation of vascular smooth muscle cells initiated normally in homozygous null mice, but failed to progress. By E9.5, extensive vascular smooth muscle cell development was observed throughout the vasculature of wildtype mice, but was absent in homozygous null animals. The presence of vascular smooth muscle cells in the vasculature of wildtype mice correlated with a mature vascular network, whereas the lack of these cells in the homozygous null animals correlated with a leaky and dysplastic vasculature. Examination of vascular smooth muscle cell development in the yolk sacks of homozygous null revealed similar results, where deficient vascular smooth muscle cell investment along the vascular plexus preceded defects in vascular network remodeling. In contrast to homozygous null mice, heterozygous null animals were viable to adulthood; however, these mice developed clinical manifestations of hereditary hemorrhagic telangiectasia, developing skin telangiectases and nosebleeds (Bourdeau et al., 1999). These results demonstrate that endoglin is required for vessel integrity and stabilization, and suggest that endoglin does this by positively regulating the differentiation or function of vascular smooth muscle cells as opposed to controlling endothelial cell behavior.

Results from studies of endoglin null mice suggest that endoglin executes some crucial function in the development of mural cells, though much of the studies characterizing the effects of endoglin on cell biology have been done in endothelial cells. However, such studies have proven that endoglin is required for normal endothelial cell behavior. Though the precise function endoglin performs in vascular

cell biology is still unclear, these studies have provided much insight on its ability to modulate intracellular signaling and functional output in response to TGFβ1.

Endoglin exists as a disulfide-linked homodimer on the surfaces of a variety of cell types including endothelial cells, and has two splice variants. The long (L) variant (referred to as endoglin) is more ubiquitously expressed and contains the 47 residue cytoplasmic tail, while the short (S) variant (referred to as S-endoglin) has a shorter 14 residue carboxy terminal. Interestingly, endoglin and betaglycan can associate together in TGFβ receptor complexes in endothelial cells (Wong et al., 2000)(Wong et al., 2000). Endoglin is able to interact with TGFβ1 and TGFβ3 only when either is physically associated with a heterotetrameric TβRII/ TβRI complex (Barbara et al., 1999; Cheifetz et al., 1992; Letamendia et al., 1998; Yamashita et al., 1994). However, endoglin can associate with both TβRII and ALK5 in the absence of ligand (Guerrero-Esteo et al., 2002). A region between amino acids 437 and 558 in the extracellular domain, as well as the cytoplasmic domain of endoglin interacts with TβRII; these regions also interact with ALK5. In addition, ALK5 interacts with another region within the extracellular domain. Interestingly, the interaction between endoglin and TβRII is not regulated by TβRII kinase activity, while ALK5 only associates with endoglin when in its inactive form. Therefore, endoglin homodimers exist in association with inactive TβRII homodimers, and upon ligand binding, these complexes recruit ALK5. ALK5 becomes activated and both it and TβRII phosphorylate endoglin within its cytoplasmic tail. This is followed by ALK5 dissociating from the complex. Association of endoglin with TβRII decreases TβRII

autophosphorylation but enhances the kinase activity of ALK5. This results in increased phosphorylation of SMAD2 and in SMAD2 transcriptional activity. Interestingly, other studies indicate that endoglin inhibits ALK5/SMAD2 while promoting ALK1/SMAD1 signaling, an observation that may come from differences in expression of other co-receptors between cell lines (Lee et al., 2008; Velasco et al., 2008).

In addition to regulating SMAD-induced transcription, endoglin also regulates cell adhesion and migration. Treatment of human endothelial cell monolayers with the anti-endoglin monoclonal antibodies 44G4 and RMAC8 increased adhesion of the monocyte cell line U-937 (Gougos et al., 1992). Exposure of treated cells with an RGD-containing fibronectin hexapeptide but not an RGE-containing peptide reversed the increased adhesion observed between these cell types. This experiment provided the first evidence that endoglin could modulate cell adhesion and suggested that endoglin may also regulate processes such as cell migration and cytoskeleton dynamics. Furthermore, it supported a mechanism where endoglin activity blocked cell adhesion by negatively regulating integrin function. The extracellular domain of the human endoglin protein contains and RGD motif, suggesting that this receptor interacts with integrin complexes. This motif is not conserved in mice. However, the cytoplasmic tail of endoglin does contain a conserved PDZ interaction motif which has been shown to interact with several different proteins involved in regulation of actin cytoskeleton dynamics. Endoglin interacts with the Lim protein family member zyxin-related protein-1or thyroid hormone receptor interacting protein 6 (ZRP-1 or

TRIP6, respectively) (Sanz-Rodriguez et al., 2004). Endoglin expression caused ZRP-1 to localize with actin stress fibers in endothelial cells. In the absence of endoglin, ZRP-1 was associated primarily within focal complexes. ZRP-1 has been shown to become recruited into focal adhesions and to the actin cytoskeleton in response to lysophosphatidic acid-stimulation of LPA_2 receptor in NIH 3T3 cells and fibroblasts (Lai et al., 2005). c-Src phosphorylates ZRP-1 at tyrosine 55; this results in ZRP-1 recruitment of Crk and p130Cas. Crk and p130Cas then promote activation of ERK and induce cell migration. Interestingly, integrin signaling-induced tyrosine phosphorylation of p130Cas has been shown to result in recruitment of SMAD3 to p130Cas and reduction TGFβ1-induced SMAD3 phosphorylation; the same study found that TGFβ1 induced complex formation between p130Cas, ALK5, and SMAD3 (Kim et al., 2008b). P130Cas is critical for normal cell migration, adhesion and spreading. Focal complex-associated p130Cas is phosphorylated by Src and FAK; this results in Rac1 activation and integrin clustering, culminating in actin polymerization, lamellapodia extension, and cell migration (Burridge and Wennerberg, 2004). ZRP-1 knockdown in HeLa cells demonstrated that ZRP-1 was required for maturation of focal complexes into focal adhesions, formation of actin stress fibers, and reorganization of the actin cytoskeleton at sites of cell to cell contact (Bai et al., 2007). Furthermore, knockdown of ZRP-1 in these cells resulted in reduced FAK phosphorylation, increased Rac1 activity, and aberrant actin polymerization, suggesting that ZRP-1 coordinates cell contact signals with actin polymerization to allow for cell migration. Endoglin's ability to interact with and spatially regulate ZRP-

1, which binds other adaptor proteins important in contact-induced signaling, suggests that endoglin can control focal adhesion signaling though controlling focal adhesion composition. Endoglin was also found to interact with zyxin via its cytoplasmic tail (Conley et al., 2004). Indeed, expression of endoglin reduced the level of focal adhesion-associated zyxin, p130Cas, and Crk in human endothelial cells, consistent with the observed increase in localization of ZRP-1 with endothelial actin stress fibers seen in response to overexpression of endoglin (Sanz-Rodriguez et al., 2004).

The presence of endoglin in focal adhesions is associated with a reduction in focal adhesion-associated p130Cas, ZRP-1, zyxin, and Crk, as well as a reduction in cell migration. One possibility is that endoglin competes with or disrupts interactions with other "pro-migratory" binding partners of zyxin and ZRP-1, thus preventing their incorporation into higher order "pro-migratory" complexes. Endoglin's cytosolic domain interacts with LIM domains within zyxin and ZRP-1 (Conley et al., 2004; Sanz-Rodriguez et al., 2004). LIM domains are cysteine-rich domains that mediate protein-protein interactions and are common in proteins found in focal adhesions (Wang and Gilmore, 2003). The LIM domains of zyxin and ZRP-1 have been shown to regulate interaction of these proteins with several regulators of cytoskeletal-dynamics in addition to p130Cas and Crk, including VASP, CRP, α-actinin, Lats, and Grb2 (Beckerle, 1997). As endoglin presumably disrupts p130Cas/Crk interactions with zyxin and ZRP-1, it is reasonable to hypothesize that there is a concomitant decrease in other associated proteins as well. Furthermore, endoglin also interacts with RGS19-interacting protein 1 (GIPC) via its cytoplasmic tail (Lee et al., 2008). Indirect

immunoflourescent staining of GIPC and endoglin revealed that these proteins associate in focal complex-like plaques located at the periphery of endothelial cells. Expression of GIPC inhibited endothelial cell migration and knockdown of GIPC reduced TGFβ1-induced SMAD1 phosphorylation, both in an endoglin dependent manner. GIPC regulates surface turnover of several receptors including betaglycan, and was also found to enhance surface levels of endoglin (Ray et al., 2010). All together, these observations suggest that in endothelial cells, endoglin is stabilized at sites of focal complex formation by GIPC; this leads to enhanced TGFβ1-induced R-SMAD phosphorylation, disruption of "pro-migratory" zyxin/ZRP-1 complexes, and reduced cell migration.

In the current study, I show for the first time, that endoglin is expressed by and functionally significant to primary pericytes and 10T1/2 cells. Immunoprecipitation of endoglin, ALK5, or TβRII from 10T1/2 cell lysates revealed that SPARC specifically associates with endoglin (Fig. 2.5 B). This interaction appears to be direct as binding between endoglin and SPARC was also observed in a cell free system (Fig. 2.5 C). For these assays, a Fc-fusion form of endoglin was used which consisted of the extracellular domain of endoglin fused to an Fc domain. The observed interaction between SPARC and this Fc-endoglin fusion protein suggests that the extracellular domain of endoglin is sufficient for its capacity to interact with SPARC. Endoglins capacity to associate with focal complexes prompted me to assess focal complexes in *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes for the presence of endoglin. I was surprised to find that endoglin associated with plaque-like structures located at

the cell periphery of *SPARC*$^{-/-}$ but not *SPARC*$^{+/+}$ pericytes (Fig. 2.6 D). These structures were enriched for vinculin, identifying them as focal complexes. The formation and maturation of focal complexes is associated with the recruitment of FAK into laterally-clustered integrins, where, as stated above, it coordinates many of the intracellular signaling events required for normal cell migration. To further verify that endoglin was associated with functional focal complexes, I examined endoglin immune complexes for the presence of FAK (Fig. 2.6, B). Focal complex formation requires cell contact with an adhesive substrate; therefore, I compared endoglin-associated FAK levels from pericytes that were suspended in growth media with endoglin-associated FAK levels from pericytes allowed to adhere to tissue culture grade plastic or fibronectin. I found that FAK was recruited into endoglin complexes of *SPARC*$^{-/-}$ pericytes only when the cells were allowed to adhere to a substrate, demonstrating that endoglin associates with functional focal complexes. The observation that endoglin associated with focal complexes in primary *SPARC*$^{-/-}$ but not *SPARC*$^{+/+}$ pericytes, strongly suggests that the interaction between SPARC and endoglin negatively regulates endoglins capacity to incorporate into focal complexes. As recruitment of endoglin into focal complexes has previously been demonstrated to be associated with elevated TGFβ1 receptor signaling, the aberrant localization of endoglin in *SPARC*$^{-/-}$ pericytes correlated with the elevated basal transcription of *CTGF*, *BIGH3*, and *PAI-1*, and increased basal SMAD2 phosphorylation as expected (Fig. 2.4, B and C). Therefore, I asked whether endoglin was required for the increased basal TGFβ1 signaling in these cells. I was unable to detect differences in

expression levels of endoglin between *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$; therefore, any differences in SMAD2 phosphorylation would suggest that aberrant localization of this receptor was responsible for its effect on SMAD2 (Fig. 2.4 B). I found that transfection of *SPARC*$^{-/-}$ pericytes with endoglin shRNA but not control was indeed sufficient to decrease TGFβ1 receptor activity (Fig. 2.6 C). Knockdown of endoglin in *SPARC*$^{+/+}$ pericytes had no effect on SMAD2 phosphorylation, demonstrating that endoglin was mediating TGFβ1 receptor activity in pericytes only in the absence of SPARC and suggesting that SPARC blocked endoglin from facilitating some mechanism of TGFβ activation within focal complexes.

Focal complex-associated endoglin has also been associated with decreased cell migration; therefore, as focal complex-associated endoglin correlated with increased SMAD2 phosphorylation in *SPARC*$^{-/-}$ pericytes, I expected that, in the absence of SPARC, endoglin was impairing pericyte migration. To test if this was true, I assessed whether endoglin negatively regulated 10T1/2 cell migration and if this was dependent on SPARC. I found that knockdown of endoglin had no effect on 10T1/2 cell migration; however, knockdown of endoglin reversed the inhibitory effect of SPARC knockdown on migration (Fig. 2.6 D). I then examined whether knockdown of endoglin could also reverse the inhibitory effect of neutralizing extracellular SPARC, which presumably interacts with the extracellular region of endoglin, on 10T1/2 cell migration (Fig. 2.5 C; 2.6 D). Neutralization of extracellular SPARC with the monoclonal antibody reduced migration of 10T1/2 cells treated with control shRNA, but had no effect on cells transfected with endoglin shRNA. These

findings suggest that SPARC binds to endoglin on the surfaces of pericytes to block endoglin-mediated impairment of pericyte migration.

These observations demonstrate that endoglin is able to regulate pericyte behavior and that regulation of this activity requires SPARC. Endoglin-mediated effects on TGFβ1 receptor activity and pericyte migration were only observed upon depletion of cellular SPARC, supporting a mechanism where endoglin associates with focal complexes to promote TGFβ1-induced activity, which includes SMAD2 phosphorylation and blockade of pericyte migration, and that SPARC functions to negatively regulate this process. Interestingly, I found that knockdown of endoglin did not affect the migration of the pericyte-like 10T1/2 cell line, or SMAD2 phosphorylation in *SPARC*$^{+/+}$ pericytes, but others have found that endoglin deficiency in endothelial cells, in the absence of any manipulation of SPARC levels, is sufficient to impair their migration. This may be due, in part, to differences in levels of expression of SPARC or endoglin between endothelial cells and pericytes. I found that SPARC expression was localized to the microvasculature of normal adult mouse pancreata, and, importantly, was most apparent in NG2^{+} pericytes (Fig. 2.1 B). One possibility is that the lower level of SPARC secreted by endothelial cells is not sufficient to block endoglin activity; therefore, in these cells, endoglin has a persistent negative effect on migration and TGFβ1 receptor activity. It is also possible that endothelial cells express higher levels of endoglin compared to pericytes. This is plausible as the overexpression of endoglin has been demonstrated to impair the migration of a variety of cell types. Another possibility is that SPARC is secreted with

endoglin in pericytes and not in endothelial cells, thereby constitutively inhibiting endoglins affect on cell behavior. Indirect immunofluorescent staining of endoglin and SPARC in primary pericytes revealed that these proteins co-localize in what appears to be the golgi and endoplasmic reticulum, as well as in distinct punctate structures which may represent vesicles near the cell periphery (Fig. 2.5 A). In this scenario, endoglin is presented at the cell surface already bound to SPARC; therefore, for endoglin to inhibit pericyte migration and promote TGFβ1 receptor activity, SPARC must somehow become dissociated from it.

The next question becomes how does endoglin incorporation into focal complexes result in enhanced TGFβ1 activity? Endoglin itself does not exhibit kinase activity, and though recruitment of endoglin into focal complexes has previously been shown to enhance TGFβ1 signaling, it is clear that other factors must be involved.

Regulation of αV integrin-mediated TGFβ activation by SPARC

αV integrins are one of the most widely studied group of integrins involved in angiogenesis, yet the regulation of their activity is not completely understood (Hynes 2007). αV integrin knockout mice have revealed that this subunit is required for perinatal survival (McCarty 2002). These mice exhibited aberrant association of cerebral blood vessels with the surrounding parenchyma. Many studies have revealed that αV integrin expression and activity becomes induced in vascular cells in response to various angiogenic stimuli (Brooks et al., 1994; Hynes, 2007). These integrins can dimerize with β3, β5, or β8 subunits to form vitronectin receptors, and with the β6

subunit to form a fibronectin receptor. Expression of αVβ3 integrin was first found to be expressed by angiogenic vascular tissue within dermal wounds (Brooks et al., 1994). Expression was also found to increase during angiogenesis using the chick chorioallantoic membrane assay. Importantly, incubation of the chorioallantoic membrane with a monoclonal αVβ3-blocking antibody blunted angiogenesis induced by FGF2 and TNF-α. Many reports demonstrate αVβ3 and αVβ5 expression becomes induced in endothelial cells following exposure of the preexisting vasculature to an angiogenic stimulus; however, I and others have found that stellate cells, which function as pericytes in various organs including the pancreas and the liver, express both αV integrin and several αV integrin-binding β subunits including β3, β5, β6, and β8 (Beer et al., 2008; Somanath et al., 2009b). Specific antagonism of αvβ3 and αvβ5 has proven to be an effective strategy for blocking tumor angiogenesis in clinical trials with glioblastoma patients (Desgrosellier and Cheresh, 2010) and studies with of human dental pulp cells and various tumor lines have shown that SPARC is able to modulate expression and activity of these integrins.

In addition to interacting with the extracellular microenvironment and mobilizing the intracellular machinery to facilitate cell migration, αV integrins can also activate latent TGFβ1. αV integrins interact with the LAP found on the latent TGFβ1 complex, presumably via its RGD motif. In a mechanism that requires cell traction, some of these integrins bind to the LAP, inducing a conformational change in the latent complex that exposes the active TGFβ1 dimer without proteolytic cleavage, thus making it accessible to TGFβ receptors on adjacent cells. Via a separate

mechanism, other αV integrins interact with the LAP RGD motif and induce TGFβ1 activation; here, activation of the latent TGFβ1 complex is achieved via MMP14-mediated release of the active TGFβ1 dimer from the latent complex.

αVβ6 integrin-mediated activation of latent TGFβ1in the absence of proteolysis was initially described as a mechanism for regulating pulmonary fibrosis and inflammation, where αVβ6 integrin was found to be required for bleomycin-induced pulmonary fibrosis in mice (Munger et al., 1999). Activation of latent TGFβ1 was shown to occur in the presence of a plethora of protease inhibitors but not in the presence of a TGFβ1-neutralizing antibody or exogenous LAP. This initial study also provided several important points of insight into the mechanism responsible for this activation. LAP was first shown to interact with αVβ6 integrin using affinity chromatography where LAP was crosslinked to sepharose beads and incubated with a secreted form of αVβ6 integrin (Weinacker et al., 1994). In support of this, adhesion of β6 integrin-transfected cells to immobilized LAP was shown to increase with increasing concentrations of LAP. Importantly, cell adhesion was abolished in these experiments when LAP was replaced with a mutant form in which the RGD motif was mutated to RGE, thus demonstrating the requirement of the RGD motif within LAP. One important question was whether αVβ6 integrin required other cellular factors to activate latent TGFβ1. Analyses of mutant forms of αVβ6 integrin lacking different cytoplasmic residues within the β6 subunit identified 2 mutants that interacted with LAP as well as wildtype αVβ6; however, only one of these mutants was able to

activate latent TGFβ1. Interestingly, this mutant localized to focal adhesions, suggesting that αVβ6 may cooperate with other factors found in focal adhesions in order to activate LAP. Importantly, this possibility should not be limited to cytoplasmic factors; however, association of αVβ6 integrins with focal adhesions suggested that activation of TGFβ1 may be regulated by the actin cytoskeleton; therefore, the authors asked whether an intact cytoskeleton was required for activation. Indeed, addition of cytochalasin D, which disrupts the actin cytoskeleton, blocked activation of latent TGFβ1 without interfering with binding of latent TGFβ1 to the cell surface. αVβ6-mediated activation was latter shown to activate TGFβ3 in a similar mechanism, and activation of latent TGFβ1 was later found to be dependent on the capacity of the latent complex to associate with the ECM via LTBP1 (Annes et al., 2004; Annes et al., 2002). Importantly, these studies utilized epithelial cells for all experiments; therefore, αVβ6 is responsible for activating latent TGFβ1 in epithelial cells. Recently, αVβ5 integrin was demonstrated to activate latent TGFβ1 in myofibroblasts via a similar mechanism that was independent of proteolysis but was dependent on contraction of the actin cytoskeleton (Wipff et al., 2007). Interaction of αVβ5 with glycophosphatidylinositol (GPI)-anchored surface protein Thy-1 disrupts the LAP/αVβ5 interaction, thus preventing contraction-induced activation of latent TGFβ1 (Zhou et al., 2010). These results demonstrate a mechanism of TGFβ1 activation that entails secretion of LTBP1/LAP/TGFβ1 (the LLC), incorporation of the LLC into the ECM via LTBP1, recognition of the LLC by focal adhesion

associated-αVβ6 or αVβ5 via the LAP RGD motif, then traction-induced presentation of the active TGFβ1 dimer to its cognate receptors.

In contrast to this protease-independent, traction-induced mechanism of αV integrin-mediated activation of latent TGFβ1, αVβ8 integrin has been shown to activate TGFβ1 via a mechanism reliant on MMP14 activity. Like the mechanism behind αVβ5 and αVβ6 activation, αVβ8-mediated activation of latent TGFβ1 is facilitated by an interaction between the RGD motif within the LAP and the integrin heterodimer. This interaction was first observed using LAP-conjugated sepharose affinity chromatography. This method identified αVβ8 in epithelial cell lysates as a specific ligand for LAP (Mu et al., 2002). This interaction was disrupted in the presence of an RGD-containing peptide, while an analogous RGE-containing peptide had no effect. Adhesion of αVβ8-expressing cells to LAP or latent TGFβ1 was also shown to increase with increasing concentrations LAP or latent-TGFβ1. Interestingly, adhesion was greater to latent TGFβ1than it was to LAP alone, suggesting that interactions with active TGFβ1 may regulate cell adhesion, though experiments directly addressing this have not been carried out. I have found that neutralization of TGFβ or inhibition of signaling downstream of ALK5 increased both adhesion and spreading of SPARC-deficient pericytes while having no effect on wildtype pericytes, demonstrating that, under certain conditions, TGFβ and its receptors directly participate in cell adhesion. αVβ8-expressing cells were then co-cultured with a TGFβ1-reporter cell line in order to answer whether αVβ8 could facilitate activation

of latent TGFβ1. Indeed, co-cultures of these cells resulted in increased TGFβ1 activity, an effect that was blunted in the presence of either a neutralizing TGFβ antibody or a β8 integrin blocking antibody. Unlike the αVβ6-mediated activation of latent TGFβ1, which requires at least a portion of the β6 cytoplasmic domain, activation of latent TGFβ1 was observed in the complete absence of the β8 cytoplasmic tail, though activation levels were higher in the presence of full length β8. Analysis of TGFβ1 activation in the presence of various protease inhibitors revealed that this process was sensitive only to the MMP14 inhibitor GM6001. Furthermore, forced expression of MMP14 in αVβ8 integrin-expressing epithelial cells stimulated activated of latent TGFβ1. That αVβ8-mediated activation was dependent on metalloprotease activity suggested a mechanism where αVβ8 was functioning to present latent TGFβ1 to MMP14 and that upon interaction of MMP14 with the integrin/latent TGFβ1 complex, protease activity cleaved LAP and released TGFβ1 into the extracellular space. In support of this model, co-culture-conditioned media induced reporter expression in reporter cell cultures, and MMP14 co-localized with β8 integrin in focal complexes.

In addition to αVβ5, αVβ6, and αVβ8, integrins αVβ1 and αVβ3 have also been shown to interact with LAP to regulate TGFβ1 activity, though the precise mechanisms in which this occurs are not clear. αVβ1 integrin present in epithelial cell lysates was found to interact with LAP-conjugated sepharose beads (Munger et al., 1998). These cells adhered to LAP in an αVβ1 integrin-dependent manner. As

binding of the αV integrin to LAP seems to be a common first step in αV-integrin mediated activation of latent TGFβ1, it seems plausible that αVβ1 integrin also activates TGFβ1, though experimental proof of such activity has yet to be presented. αVβ3 integrin also mediates cell adhesion to LAP (Ludbrook et al., 2003). The interaction between αVβ3 and LAP requires as intact RGD motif, as mutation of these residues resulted in the inability of αVβ3 to facilitate adhesion to LAP. Forced expression of αVβ3 integrin in fibroblasts induced expression of a TGFβ1-chloramphenical acetyltransferase reporter construct under control of the α2(I) collagen promoter (Asano et al., 2005). Furthermore, antibody-mediated blockade of αVβ3 in sclerotic but not normal fibroblast reduced basal SMAD3 phosphorylation and α–SMA expression. Interestingly, αVβ3 has been shown to induce secretion of and act in concert with MMP2 and MMP9 to facilitate cell migration and invasion, yet its capacity to activate TGFβ1 does not rely on protease activity, which supports a mechanism of activation similar to that used by integrins αVβ6 and αVβ5 (Brooks et al., 1996; Ria et al., 2002; Rolli et al., 2003; Wipff et al., 2007).

In the current study, αV integrins were demonstrated to facilitate activation of latent TGFβ1 in primary pericytes but only in the absence of SPARC expression, identifying SPARC as a regulator of αV integrin-mediated activation of TGFβ1(Fig. 2.7 C). Interestingly, αV integrins promoted pericyte migration in a SPARC independent manner, as treatment with RMV-7, a pan-αV integrin-blocking antibody, reduced the number of both *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ migrating pericytes (Fig. 2.7 B).

This suggests that SPARC does not inhibit αV integrins from interacting with their substrates and that the defective migration of pericytes observed in the absence of SPARC is not due to a loss in αV integrin function; rather, SPARC specifically blocks αV integrins from interacting with the TGFβ1 signaling machinery. However, these results do not rule out the possibility that TGFβ1-activating β integrins are elevated in the absence of SPARC. Therefore, comparison of the surface levels of relevant β subunits in *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes will help to refine the role of SPARC in regulating αV integrin-mediated TGFβ1 activation.

αVβ6 integrin was detected at both the mRNA and protein levels in both *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes (Fig. 2.7 A; 3.1 B). As this integrin is a well established activator of latent TGFβ1, I was compelled to assess its contribution to the αV integrin-sensitive elevation of basal SMAD2 phosphorylation observed in *SPARC*$^{-/-}$ pericytes. αVβ6 integrin-mediated activation of TGFβ1 has previously been shown to be significantly enhanced in the presence of fibronectin, an effect due to fibronectin interacting with a hinge domain within LTBP, and as SPARC has previously been shown to negatively regulate fibronectin synthesis in endothelial cells, elevated fibronectin expression by *SPARC*$^{-/-}$ pericytes may account for an increase in αVβ6 integrin-mediated activation of TGFβ1 and subsequent SMAD2 phosphorylation (Fontana et al., 2005; Lane et al., 1992). Furthermore, I found that knockdown of SPARC in 10T1/2 cells resulted in increased fibronectin deposition (Fig. 3.1 A). The monoclonal antibody 10D5 was developed by immunizing integrin β6$^{-/-}$ mice with

keratinocytes expressing αVβ6 integrin and specifically recognizes αVβ6 integrin (Huang et al., 1998). This antibody has been demonstrated to completely block αVβ6 integrin activation of TGFβ1; however, treatment of *SPARC*$^{-/-}$ pericytes with 10D5 failed to reduce SMAD2 phosphorylation, suggesting that SPARC does not block αVβ6 integrin-mediated activation of latent TGFβ1 (Fig. 3.1 C). Recently, SPARC has been shown to interact with the β1 integrin subunit (Weaver et al., 2008). As αVβ1 integrin has been shown to directly engage LAP and is therefore implicated in regulating activation of TGFβ1, it may be that SPARCs ability to interact with β1 integrin contributes to its control of αV integrin-mediated activation of TGFβ1. Future studies assessing the contributions of relevant β integrin subunits to SPARCs capacity to control TGFβ1-mediated effects are needed to fully understand this regulatory mechanism.

SPARC controls TGFβ1-induced activity by regulating endoglin/αV integrin complex formation

SPARC negatively regulates TGFβ1 signaling by preventing both endoglin and αV integrin-mediated SMAD2 phosphorylation in pericytes. My results suggest that this is through a mechanism that involves blocking activation of endogenous latent TGFβ1; first, I was unable to detect any differences in total TGFβ1 mRNA, cell associated TGFβ1 protein, or secreted TGFβ1 protein between *SPARC*$^{-/-}$ and *SPARC*$^{+/+}$ pericytes, suggesting that the increase in basal TGFβ1-induced transcription

and SMAD2 phosphorylation in *SPARC*$^{-/-}$ pericytes was due to enhanced activation and not increased production of TGFβ1 (Fig. 2.4, A and B). In support of this, analysis of active TGFβ1 protein associated specifically with the extracellular cell surface revealed that more active TGFβ1 was associated on the surfaces of *SPARC*$^{-/-}$ pericytes (Fig. 2.4 D). Second, knockdown of TGFβ1 expression reduced SMAD2 phosphorylation in *SPARC*$^{-/-}$ pericytes to a level similar to that observed in *SPARC*$^{+/+}$ pericytes, demonstrating that endogenous TGFβ1, though present, does not contribute to basal TGFβ1 receptor activity in the presence of SPARC, and that the TGFβ1 receptors in both *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes exhibit similar basal sensitivity to exogenous stimulation (Fig. 2.4 C). Third, shRNA-mediated knockdown of endoglin in *SPARC*$^{-/-}$ pericytes also reduced SMAD2 phosphorylation to levels observed in *SPARC*$^{+/+}$ pericytes (Fig. 2.6, C). Lastly, neutralization of αV integrins, well established facilitators of latent TGFβ1 activation, mediate SMAD2 phosphorylation in *SPARC*$^{-/-}$ but not *SPARC*$^{+/+}$ pericytes (Fig. 2.7 C). As αV integrins are present in focal complexes, the question then becomes whether endoglin and αV integrins interact in the absence of SPARC. I hypothesized that, due to the increase in basal TGFβ1 activity in *SPARC*$^{-/-}$ pericytes, endoglin and αV integrins would be associated in focal complexes, as TGFβ1 has been shown to induce ALK5/SMAD3/p130Cas complex formation in both fibroblasts and epithelial cells (Kim et al., 2008). Also, TGFβ1 has been shown to induce TβRII/αVβ3 integrin complex formation in both fibroblasts and epithelial cells, and this association enhanced TGFβ1 signaling

(Galliher and Schiemann, 2006; Scaffidi et al., 2004). As endoglin interacts with both TGFβ1 receptors and various focal complex-associated proteins, it may serve to bridge the gap between TGFβ signaling and αV integrin activity. SPARC may function to regulate the spatial distribution of TGFβ1 receptors on the cell surface through its interaction with endoglin; if endoglin serves to bridge TGFβ1 receptors to αV integrins and TGFβ1, SPARC may block endoglin from incorporating into αV integrin-containing focal complexes, thus not giving TGFβ1 receptors access to αV integrin-associated TGFβ1. In this proposed mechanism, SPARC attenuates αV integrin-mediated TGFβ1-induced responses by interacting with endoglin and blocking TGFβ1 receptor recruitment into focal complexes. Indeed, endoglin does not exhibit kinase activity, so the finding that it can induce SMAD2 phosphorylation in the absence of SPARC suggest that somehow, endoglin is promoting activation of type I and II TGFβ receptors. In support of this proposed mechanism, I found that αV integrins were enriched in endoglin immune complexes from *SPARC*$^{-/-}$ pericyte lysates compared to lysates harvested from *SPARC*$^{+/+}$ pericytes (Fig. 2.7 D). This interaction did not require formation of focal complexes, as αV integrin was present in endoglin immune complexes from cells in suspension and cells adhered to tissue culture-grade and fibronectin-coated plastic at similar levels, suggesting that this association does not rely on intracellular signaling that occurs in maturing focal complexes. Furthermore, recombinant SPARC decreased the association of αV integrins with these complexes (Fig. 2.7 E). Therefore, the interaction of SPARC with

endoglin and endoglin with αV integrins represents a novel signaling unit used by pericytes to modulate their response to TGFβ1.

Future directions

Altogether, the results presented here describe a novel mechanism used by pericytes to regulate activation of TGFβ1, where SPARC restricts endoglin and αV integrin-mediated TGFβ1-induced responses. Proper regulation of TGFβ1 activation is critical for stabilization of a nascent vascular network. αV integrin-mediated activation of TGFβ1 provides a mechanism that can account for spatial and temporal control of TGFβ1-induced pericyte responses during angiogenesis because only cells that are expressing the prober integrin subunits are able activate TGFβ1. My findings now add a requirement for endoglin in αV integrin-mediated activation of latent TGFβ1 in pericytes. αV integrin-mediated regulation of TGFβ1 activation has been demonstrated to be responsible for bleomycin-induced fibrosis in mice (Hogmalm et al., 2010; Munger et al., 1999). Also, studies in β8 integrin knockout mice have revealed a requirement of this integrin subunit for vascular development. β8 integrin exclusively associates with the αV subunit; therefore effects arising from β8 integrin deficiency are due to deficient αVβ8 activity. Global knockout of β8 integrin results in insufficient vascularization of the yolk sac and placenta, likely due to a deficiency in vascular stabilization (Zhu et al., 2002)(Zhu et al., 2002). Most of these mice die during development, but some survive and die shortly after birth, exhibiting extensive

cerebral hemorrhaging. αVβ8 integrin may also be relevant to the activation of TGFβ1 that occurs in *SPARC*$^{-/-}$ pericytes, as inhibition of αVβ6 activity did not blunt phosphorylation of SMAD2 in these cells; furthermore, *SPARC*$^{-/-}$ pericyte surfaces contained active (12.5 kDa) TGFβ1 protein, suggesting that MMP activity is contributing to the enhanced endoglin/αV integrin-dependent TGFβ1 activity. Inhibition of MMP2 and MMP9 failed to reduce SMAD2 phosphorylation as well, suggesting that the responsible MMP is MMP14, as it is the only MMP demonstrated to be required for αV integrin-mediated activation of latent TGFβ1 (Fig. 8.2). Recently, MMP14 has been implicated in regulating vascular homeostasis via a mechanism dependent on ALK5 and TGFβ1 (Sounni et al., 2010). Neutralization of TGFβ1 and ALK5 inhibition was sufficient to induce blood vessel leakage in the Miles assay, an in vivo assay used to measure the response of quiescent cutaneous blood vessels to mustard oil (Miles and Miles, 1952). Importantly, the authors observed that this response phenocopied inhibition of MMP14 with GM6001. The authors also found that collagen mutant mice exhibited enhanced MMP14 activity and were less susceptible to mustard oil induced vascular leakage, and that this effect was due to activation of TGFβ1.The authors speculated that the enhanced vascular leakage observed in response to MMP14 or ALK5/TGFβ1signaling inhibition was due to disrupted TGFβ1 signaling specifically in pericytes and smooth muscle cells. This is in agreement with the observation that ALK5 deletion results in the failure of smooth muscle cell layers to become established during mouse development (Seki et al., 2006). Activation of TGFβ1 by pericytes is proposed to occur upon contact of

migrating pericytes with the nascent endothelial tube. TGFβ1 then stimulates pericyte secretion of basement membrane proteins and inhibits further pericyte migration; therefore, activation of TGFβ1 must occur specifically at the pericyte/endothelial cell interface. One possibility is that αV integrin-expressing pericytes encounter latent TGFβ1 associated with endothelial cells; these cells then activate TGFβ1, which then induces pericyte differentiation, that is, the secretion of basement membrane proteins and prevention of further migration. Our data suggests that SPARC acts as a negative regulator of such a mechanism, blocking association of αV integrins with the TGFβ1 signaling machinery, thus facilitating cell migration.

Pericyte recruitment is a required step of the angiogenic cascade. Multiple signaling molecules including PDGF–BB, Ang-1, and S1P, act together to induce mobilization of pericytes to nascent vessels. TGFβ1 signaling at the endothelial cell/pericyte interface then blocks pericyte migration and induces basement membrane deposition. TGFβ1 signaling is also required for maintaining contact between endothelial cells and pericytes. Vessel-associated pericytes are able to provide growth factors such as VEGF-A to the underlying layer of endothelial cells via gap junctions. Failure for pericytes to become properly associated with nascent blood vessels results in destabilization of the nascent vasculature. Morphologically, such vascular networks appear highly abnormal, and in addition to lacking pericyte coverage, they exhibit tortuous vessels and areas of endothelial cell hyperplasia and vessel hemorrhaging. Such vessels result from angiogenesis occurring in solid tumors; therefore, the

functional significance of pericytes to tumor vasculature is a clinically relevant area of basic research.

Targeting angiogenesis has become a standard therapy for a variety of solid tumors (Holash et al., 2006). Such therapies reduce the number of tumor blood vessels by targeting VEGF-A. This results in the endothelial cell apoptosis and control of tumor growth. This effect relies in part on the bulk tumor cells being sensitive to a hypoxic environment. Recently, the functional significance of pericytes in the tumor vasculature has been examined by selectively targeting these cells in various experimental models. Targeting PDGFRβ, which is specifically expressed by pericytes, resulted in reduction of blood vessel number and control or reduction in tumor size in models of islet, colon, and prostate cancers (Bergers et al., 2003; Ozerdem, 2006; Reinmuth et al., 2001; Shaheen et al., 2001). The resulting tumor vasculatures were comprised of vessels that lacked pericyte coverage and exhibited hyperdilation. These findings highlight the significance of tumor-associated pericytes to maintaining a functional, though abnormal, vasculature, and provide reason for furthering the understanding of the mechanisms involved in regulating pericyte behavior. The current study identifies SPARC as a novel inhibitor of endoglin/αV integrin mediated TGFβ1 activation on pericytes. This conclusion provides rationale for the hypothesis that targeting SPARC would inhibit pericyte recruitment in tumors. This is supported by my finding that, in a transgenic model of PDAC, pericyte recruitment was significantly reduced in tumors from *SPARC*$^{-/-}$ PDAC mice compared to *SPARC*$^{+/+}$ PDAC mice (Fig. 2.1 F). Deficient pericyte recruitment in *SPARC*$^{-/-}$

tumors has also been previously reported (Arnold et al., 2010; Puolakkainen et al., 2004). Our lab also found that pericyte recruitment was enhanced in tumors overexpressing MMP9, an established activator of pericyte mobilization; this pericyte phenotype was dependent on the presence of SPARC, as tumors grown in *SPARC*$^{-/-}$ mice failed to exhibit any increase in pericyte recruitment, underscoring the requirement of SPARC for normal pericyte migration (Fig. 3.3).

One of the key functions of pericyte coverage in a vascular network is to provide growth factors to the underlying endothelium; these in turn induce survival signals that result in sustained vessel viability. One hypothesis is that in the presence of pericytes, vessels are not susceptible to depletion of exogenous growth factors in terms of their viability, and that in the absence of pericyte coverage; they become reliant on exogenous growth factors for their viability. Tumor vessels are thusly said to be subject to a process termed vessel normalization; this is a regression of pericyte deficient blood vessels induced by the depletion of exogenous growth factors. This hypothesis is supported by the observation that anti-VEGF-A therapy selectively ablates tumor vessels that lack pericyte investment in many models of solid cancers (Jain, 2005). The resulting vasculature is comprised of vessels that exhibit pericyte coverage resembling, a normal vascular network. These vessels are less susceptible to VEGF-A depletion and are more efficient at delivering oxygen to the bulk of the tumor. It is reasonable to hypothesize that if pericytes were targeted in conjunction with endothelial cells, this would result in fewer vessels becoming normalized as they would be susceptible to exogenous VEGF-A depletion. This would in turn result in a

more of the tumor mass being unable to receive oxygen from the blood. A preclinical study in mice revealed that, indeed, targeting both endothelial and pericyte compartments of the tumor vasculature resulted in an enhanced clearance of blood vessels and an enhanced reduction in tumor size compared to either treatment alone (Bergers et al., 2003). The benefits of such a strategy would have to be weighed against the benefits of targeting endothelial cells alone. As normalized vessels facilitate increased penetration of chemotherapeutics, thus increasing the efficacy of drug therapy, the decision to target both pericytes and endothelial cells must be based on the tumors capacity to survive a hypoxic environment; nevertheless, identification of SPARC as a novel regulator of pericyte migration provides a novel target for pericyte recruitment, the manipulation of which may be prove to be a beneficial anti-angiogenic strategy.

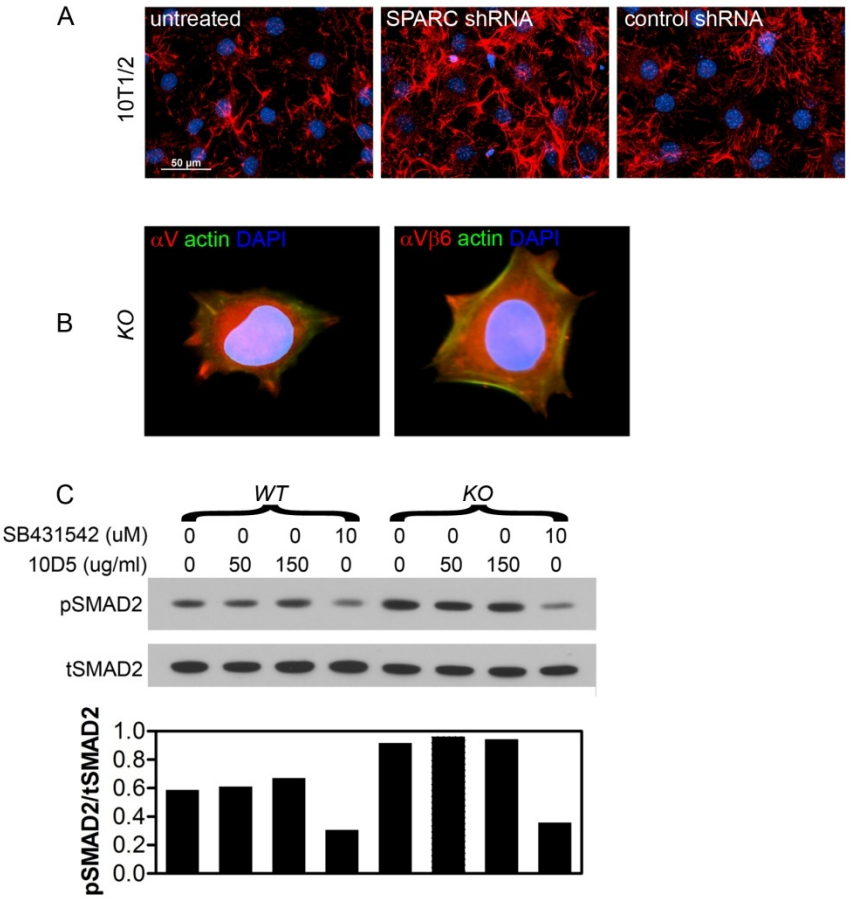
Figure legends

Figure 3.1. **αVβ6 integrins do not mediate SMAD2 phosphorylation in *SPARC*$^{-/-}$ pericytes**. (A) Knockdown of SPARC enhances fibronectin deposition in 10T1/2 cells. 10T1/2 cells were transfected at approximately 70% confluency with either SPARC shRNA or non-targeting control shRNA expression constructs from Sigma Mission. 16 hours later, cells were trypisinized and seeded at confluency onto silane-coated chamber slides along with mock-transfected cells (untreated). Cells were then cultured for 32 hours in the presence of 5% fetal bovine serum-supplemented DMEM at 37^{o} C. Cells were then fixed in chilled acetone for 5 minutes, then stained using anti-fibronectin IgG primary antibody, and Cy3-conjugated secondary IgG (red channel). Nuclei were visualized with DAPI (blue channel). (B) αVβ6 expression in *SPARC*$^{-/-}$ pericytes. *SPARC*$^{-/-}$ pericytes were serum starved overnight then seeded at low density onto fibronectin-coated chamber slides and allowed to adhere for 1 hour. Cells were then fixed and as in (A) and stained using antibodies against αV integrin (right panel) or αVβ6 integrin (left panel). Actin cytoskeleton was visualized using phalloidin (green channel) and nuclei were visualized using DAPI (blue channel). (C) αVβ6 blockade with 10D5 does not reduce basal SMAD2 phosphorylation in *SPARC*$^{-/-}$ pericytes. Primary pericytes were seeded onto fibronectin-coated tissue culture plates at approximately 70% confluency in the presence of the indicated concentrations of the αVβ6 integrin-blocking antibody 10D5 for 17 hours in 1.5% fetal bovine serum-supplemented DMEM at 37^{o} C. Lysates were prepared as described in Chapter 2 and subjected to SDS-PAGE and western blot.

Figure 3.2. **(2*R*)-[(4-Biphenylylsulfonyl)amino]-*N*-hydroxy-3-phenylpropionamide (BiPS), an inhibitor of MMP2 and MMP9, does not reduce basal SMAD2 phosphorylation in *SPARC*$^{-/-}$ pericytes**. (A) BiPS decreases collagenase activity of pericyte-conditioned media on DQ gelatin. *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes were seeded at approximately 70% confluency in a 6 well culture dish in 1.5% fetal bovine serum-supplemented DMEM. Cells were cultured for 24 hours at 37o C in the presence of the indicated concentrations of BiPS. After 24 hours, conditioned media was obtained and assayed for collagenase activity using 1% DQ gelatin. Flourescence was measured of each sample in triplicate, and 100% activity was taken as the mean of values from non-treated samples (data not shown). (B) BiPS reduces MMP2 activity, and, to a lesser extent, MMP9 activity in *SPARC*$^{-/-}$ pericyte conditioned media using in gel zymography. Conditioned media from (A) was used for in gel zymography using gelatin as a substrate. Identification of protease species was based on molecular weight of cleavage bands. (C) BiPS does not reduce SMAD2 phosphorylation in *SPARC*$^{-/-}$ pericytes. Lysates from (A) were subjected to SDS-PAGE and western blot as described in Chapter 2. *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$.

Figure 3.3. **The absence of host SPARC inhibits MMP9-induced pericyte recruitment in an orthotopic model of pancreatic cancer**. Pan02 cells stably transfected with empty vector or with MMP9 (PRV and MMP9 respectively) were injected into *SPARC*$^{+/+}$ or *SPARC*$^{-/-}$ mice and allowed to develop into tumors for 8 weeks. Tumors were then harvested, sectioned, and stained to visualize blood vessels and pericytes. A representative tumor section stained for blood vessels (MECA32,

red) and pericytes (α-SMA, green) (left panel). Quantification of pericyte-associated vessels (PAVs)s in tumors grown in *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ mice. PAV number was manually assessed in each group and expressed as average PAVs/ field + standard error margin (SEM). **P* < 0.005 versus *WT* PRV, ***P* < 0.0009 versus *WT* PRV. *WT* = *SPARC*$^{+/+}$, *KO* = *SPARC*$^{-/-}$.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10T1/2
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50 µm
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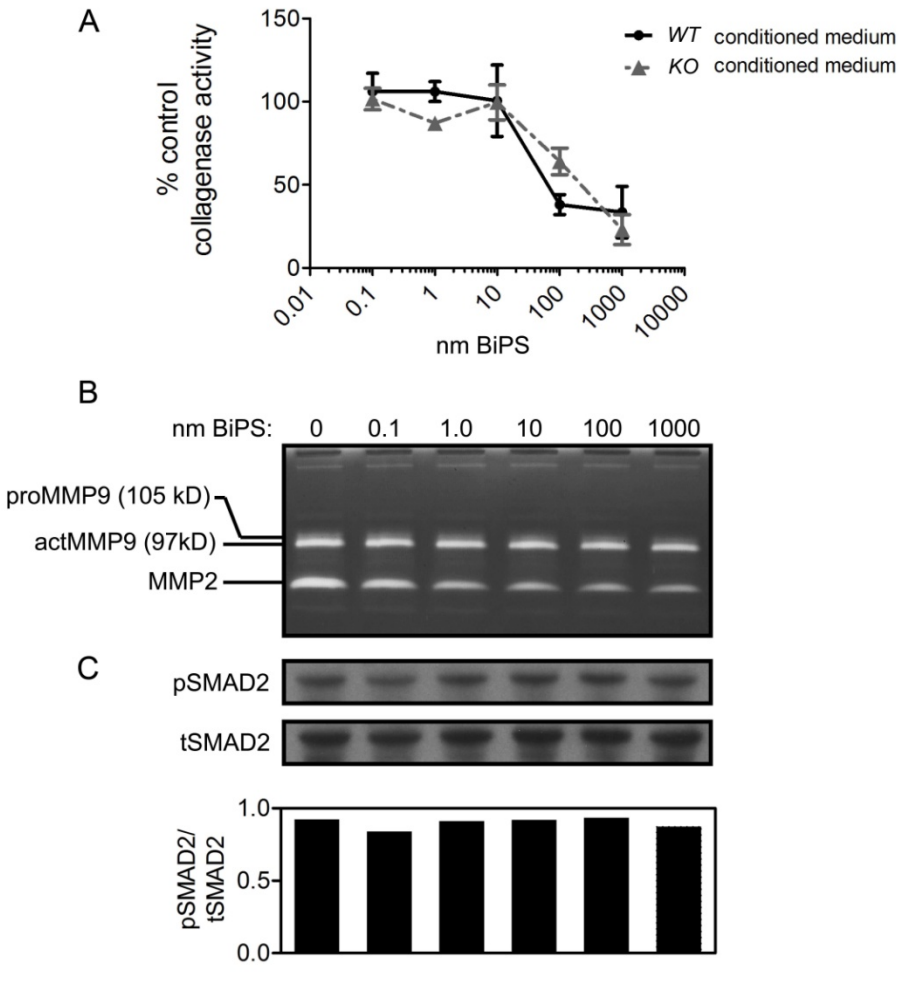
C
WT
KO
SB431542 (uM) 0 0 0 10 0 0 0 10
10D5 (ug/ml) 0 50 150 0 0 50 150 0
pSMAD2
tSMAD2
pSMAD2/tSMAD2
1.0
0.8
0.6
0.4
0.2
0.0

FIGURE 3.1


A
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150
100
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nm BiPS
WT conditioned medium
KO conditioned medium
B
nm BiPS: 0 0.1 1.0 10 100 1000
proMMP9 (105 kD)
actMMP9 (97kD)
MMP2
C
pSMAD2
tSMAD2
pSMAD2/ tSMAD2
1.0
0.5
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FIGURE 3.2

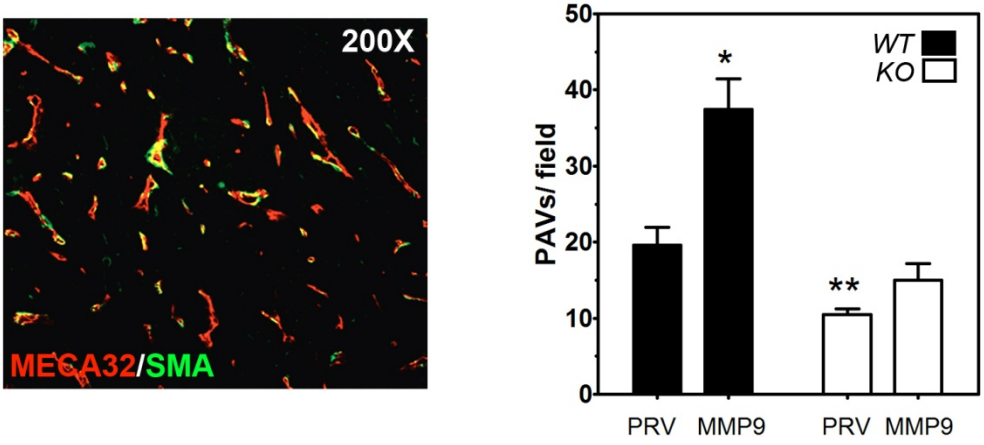
200X
MECA32/SMA
PAVs/ field
50
40
30
20
10
0
*
**
WT
KO
PRV
MMP9
PRV
MMP9

FIGURE 3.3

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