SPARC promotes pericyte recruitment via inhibition of endoglin-dependent TGF-β1 activity

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Pericytes migrate to nascent vessels and promote vessel stability. Recently, we reported that secreted protein acidic and rich in cysteine (SPARC)–deficient mice exhibited decreased pericyte-associated vessels in an orthotopic model of pancreatic cancer, suggesting that SPARC influences pericyte behavior. In this paper, we report that SPARC promotes pericyte migration by regulating the function of endoglin, a TGF-β1 accessory receptor. Primary SPARC-deficient pericytes exhibited increased basal TGF-β1 activity and decreased cell migration, an effect blocked by inhibiting TGF-β1. Furthermore, TGF-β–mediated inhibition of pericyte migration was dependent on endoglin and αV integrin. SPARC interacted directly with endoglin and reduced endoglin interaction with αV integrin. SPARC deficiency resulted in endoglin-mediated blockade of pericyte migration, aberrant association of endoglin in focal complexes, an increase in αV integrins present in endoglin immunoprecipitates, and enhanced αV integrin–mediated activation of TGF-β. These results demonstrate that SPARC promotes pericyte migration by diminishing TGF-β activity and identify a novel function for endoglin in controlling pericyte behavior.

Introduction

During angiogenesis, nascent blood vessels initially form as endothelial tubes that become coated with pericytes. Pericytes are mobilized from preexisting vessels by the combined activities of several proliferation- and migration-stimulating factors, including matrix metalloprotease 9 (MMP9) and PDGF-BB. Mobilized pericytes migrate to the newly formed endothelial tube and, upon contact, induce vessel maturation and stabilization (von Tell et al., 2006). This process is required for normal angiogenesis, as lack of adequate pericyte coverage results in vessel abnormalities, including leakiness and hemorrhaging (Lindahl et al., 1997; Hellström et al., 2001). Unlike normal vasculature, vessels within tumors are typically leaky, tortuous, and exhibit abnormal pericyte coverage (Helmlinger et al., 1997; Benjamin et al., 1999; Eberhard et al., 2000). Antiangiogenic tumor therapy is believed to be effective at treating some types of cancer by selectively ablating blood vessels that lack pericyte coverage, thereby increasing the efficiency of blood transport within the tumor, which increases the delivery of chemotherapeutics (Gerhardt and Semb, 2008). Targeting pericyte recruitment was shown to increase the efficacy of antiangiogenic tumor therapy in a mouse model of islet carcinoma (Bergers et al., 2003), demonstrating that modulation of pericyte behavior can be therapeutically 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 a matricellular protein whose expression is induced during angiogenesis (Lane et al., 1994; Iruela-Arispe et al., 1995). SPARC has been implicated in cellular processes critical to angiogenesis, including migration, proliferation, and differentiation (Kupprion et al., 1998; Francki et al., 2003; Motamed et al., 2003; Chlenski et al., 2007). The activity of SPARC appears to be indirect and relies in part on its ability to influence various growth factor signaling pathways. For example, SPARC can directly interact with VEGF-A and with PDGF-BB and PDGF-AB and prevent their interaction with cell surface receptors (Raines et al., 1992; Kupprion et al., 1998). However, SPARC

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Abbreviations used in this paper: α-SMA, α smooth muscle actin; KO, knockout; LAP, latency-associated peptide; MMP, matrix metalloprotease; PDAC, pancreatic ductal adenocarcinoma; pSMAD2, phosphorylated SMAD2; qPCR, quantitative PCR; shRNA, small hairpin RNA; SPARC, secreted protein acidic and rich in cysteine; TβRII: TGF-β receptor II; tSMAD2, total SMAD2; WT, wild type.

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antagonizes FGF2/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 orchestrates the deposition of the ECM and can modulate the interaction between cells and their substratum (Murphy-Ullrich et al., 1995; Weaver et al., 2008; Bradshaw, 2009). Using an orthotopic model of pancreatic cancer, we found that pericyte recruitment was decreased in tumors grown in SPARC-deficient mice (Puolakkainen et al., 2004; Arnold et al., 2010). Though the mechanism underlying this observation is unknown, the data suggest that SPARC facilitates pericyte behavior in vivo.

TGF-β1 is a pleiotropic cytokine expressed by vascular cells during angiogenesis. TGF-β1 is secreted in a latent complex, which must be cleaved or otherwise manipulated to expose the active protein (Derynck et al., 1986; Munger et al., 1999; Annes et al., 2004). The precise cellular responses induced by active TGF-β1 depend on the specific TGF-β receptors expressed and the level of receptor expression (Schmierer and Hill, 2007). These responses vary and include migration, apoptosis, and proliferation (Goumans et al., 2003; Guasch et al., 2007; Daly et al., 2008; Yamashita et al., 2008). TGF-β1 receptors are ubiquitous throughout all tissues; therefore, the activation of latent TGF-β1 and the bioavailability of active TGF-β1 are tightly regulated (Lyons et al., 1988; Imai et al., 1997; Saharinen et al., 1999). Active TGF-β1 inhibits pericyte migration and induces expression and secretion of basement membrane proteins; accordingly, TGF-β1 signaling in these cells is restricted during angiogenesis, occurring only upon contact with endothelial cells of newly formed vessels (Sato and Rifkin, 1989; Kojima et al., 1991; Owens, 1995). Although the mechanisms behind this regulation are not entirely clear, a plausible scenario is αV integrin–mediated control of TGF-β activation, which could occur on the surface of pericytes that have reached the nascent endothelial tube.

Pericytes express TGF-β receptor II (TβRII) and the type II TGF-β1 receptor ALK5. In addition to these signaling receptors, we show that pericytes also express the accessory receptor endoglin. Endoglin, an established regulator of TGF-β1 activity in endothelial cells, interacts with the activated TGF-β1–TGF-β receptor complex and controls endothelial cell behavior by affecting TβRII/ALK1 and TβRII/ALK5 signaling and focal adhesion assembly (Conley et al., 2004; Sanz-Rodriguez et al., 2004).

In the current study, we sought to determine the mechanism by which SPARC regulates pericyte behavior. We report that SPARC promotes pericyte migration by decreasing TGF-β1 activity. We found that SPARC is expressed by pericytes in the vasculature of the adult murine pancreas and in pancreatic ductal adenocarcinoma (PDAC), where it facilitates pericyte migration by preventing endoglin from interacting with αV integrins, thereby repressing TGF-β1 activity. SPARC's capacity to regulate pericyte recruitment highlights its function as a critical component of tissue remodeling and angiogenesis.

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 fewer α smooth muscle actin^{+} (α-SMA^{+}) cell–invested blood vessels than tumors grown in *SPARC*$^{+/+}$ mice (Puolakkainen et al., 2004; Arnold et al., 2010). 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^{+} endothelial cells of the normal adult pancreas (Fig. 1 A). NG2^{+} cells were found to express SPARC in the vasculature of the normal adult pancreas and PDAC (Fig. 1, B and C, respectively). We crossed *P48Cre:LSLKras*^{G12D}*:INK4A*$^{lox/lox}$ mice, which develop PDAC (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. 1 F). We next asked whether endogenous SPARC influenced 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. 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 (Fig. S1). We found that *SPARC*$^{+/+}$ pericytes exhibited more filopodia per cell compared with *SPARC*$^{-/-}$ pericytes when spreading on fibronectin. Focal adhesion area was also greater in *SPARC*$^{-/-}$ pericytes, whereas the overall number was decreased.

We next assayed migration toward fibronectin, a provisional matrix protein, using a transwell assay. *SPARC*$^{-/-}$ pericytes exhibited a significantly reduced capacity to migrate, a feature that was reversed by the addition of recombinant SPARC (Fig. 2 A). Recombinant SPARC did not enhance the migration of *SPARC*$^{+/+}$ pericytes, suggesting that endogenous SPARC is sufficient for optimal migration (Fig. 2 C). Furthermore, small hairpin RNA (shRNA)–mediated knockdown or IgG-mediated neutralization of SPARC in *SPARC*$^{+/+}$ pericytes reduced their capacity to migrate (Fig. 2 B). Thus, pericyte-derived SPARC mediates pericyte recruitment to blood vessels, at least in part, by promoting cell migration.

Endogenous TGF-β1 blocks migration of SPARC-deficient pericytes

SPARC can regulate TGF-β1 activity in other cell types; therefore, we assessed the contribution of TGF-β1 to the migration phenotype of *SPARC*$^{-/-}$ pericytes (Schiemann et al., 2003; Francki et al., 2004; Chlenski et al., 2007). For this, we used primary pericytes and the 10T1/2 mesenchymal cell line (Reznikoff et al., 1973). Like pericytes, 10T1/2 cells can differentiate into

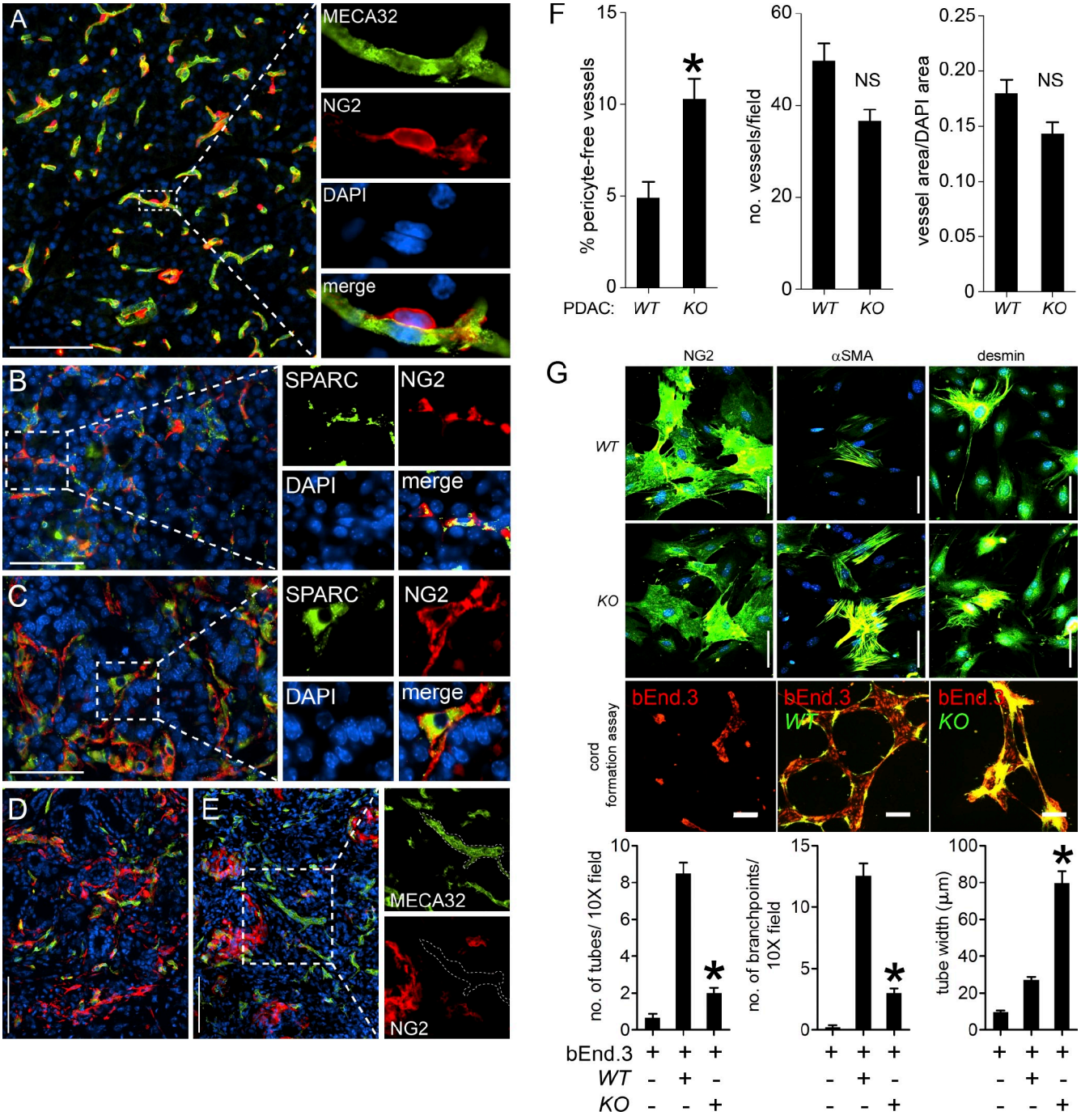

Figure 1. **SPARC is expressed by pericytes in vivo.** (A) NG2 expression is restricted to pericytes in adult mouse pancreas. MECA32 labels blood endothelial cells. The inset is magnified on the right. Bar, 100 μm. (B and C) SPARC is expressed by pericytes in pancreata from normal (B) and PDAC (p*48Cre*$^{+}$*:LSLKras*$^{G12D+}$*:INK4A*$^{lox/lox}$; C) mice. Insets are magnified on the right. (D–F) PDAC tumors in *SPARC*$^{-/-}$ animals exhibit more pericyte-free vessels. MECA32 (green) and anti-NG2 (red) immunofluorescence of *SPARC*$^{+/+}$ (D) and *SPARC*$^{-/-}$ (E) PDAC mice. An example of a pericyte-free vessel is presented in the inset of E and magnified on the right (indicated with a dotted line). (F) Percentage of pericyte-free vessels, vessel number, and relative vessel area (vessel area/DAPI area) were assessed in tumors from six *SPARC*$^{+/+}$ and eight *SPARC*$^{-/-}$ PDAC tumors. Values presented are means + SEM (≥18 20× fields per tumor; *, $P < 0.005$). (G) Isolation of primary pericytes. (top two rows) *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes express NG2 and desmin. Note that a subpopulation of these cells express α-SMA. (bottom) 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 h. The mean number of cords, number of branch points, and cord widths were quantitated from five 10× fields per well. Experiment shown is representative of three independent experiments. Errors bars represent SEM (*, $P < 0.0001$). *WT*, *SPARC*$^{+/+}$; *KO*, *SPARC*$^{-/-}$. Epifluorescent images are presented in A–E and the top two rows of G. Nikon confocal images are presented in bottom row of G (see Materials and methods).

mesenchymal lineages (Lien et al., 2006; Crisan et al., 2008; Lee et al., 2008a; Qu et al., 2008; Boeuf et al., 2009). These cells also function as pericytes when co-cultured with endothelial cells (Hirschi et al., 1998; Darland and D'Amore, 2001). Using our transwell assay, we found that TGF-β1 could inhibit both *SPARC*$^{+/+}$ pericyte and 10T1/2 cell migration (Fig. 3 A).

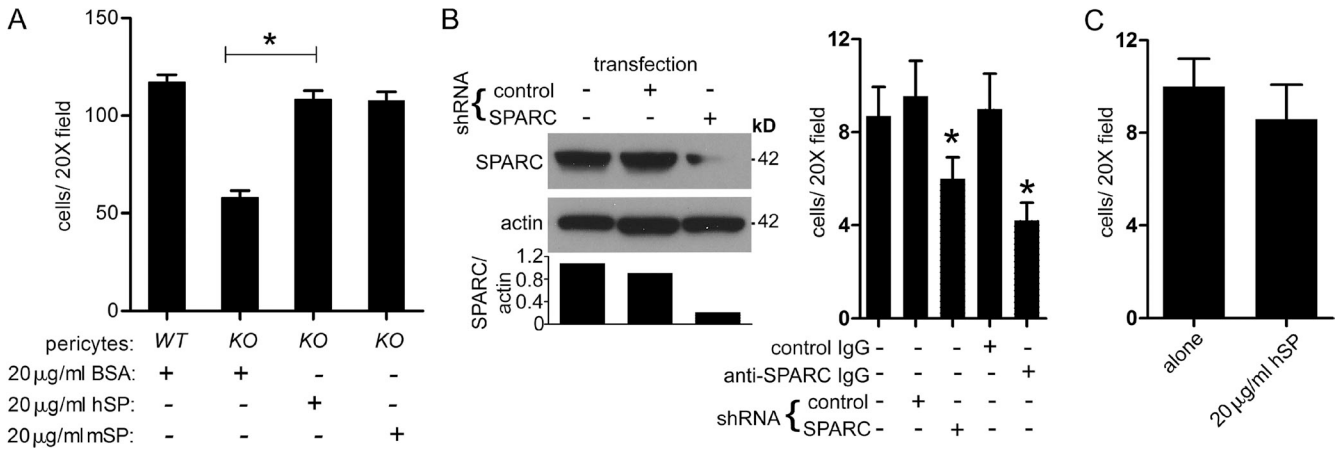

Figure 2. **Pericytes exhibit defective transwell migration in the absence of SPARC.** (A) *SPARC*$^{-/-}$ pericytes exhibit impaired migration. Cells were allowed to migrate in the presence of BSA or purified SPARC. *, P < 0.02. (B) Knockdown of SPARC in primary pericytes reduces migration. Cells were transfected with the indicated shRNA (left) 48 h before use or allowed to migrate in the presence of anti-SPARC or control IgG. *, P < 0.05 versus controls. (C) Exogenous SPARC does not affect *SPARC*$^{+/+}$ pericyte migration. Migration of *SPARC*$^{+/+}$ pericytes in the presence or absence of recombinant human SPARC (hSP) was determined. *WT*, *SPARC*$^{+/+}$; *KO*, *SPARC*$^{-/-}$; mSP, mouse SPARC. Experiments were performed in duplicate; means are represented. Error bars represent SEM.

To determine whether TGF-β was responsible for the migration phenotype of *SPARC*$^{-/-}$ pericytes, we used a TGF-β–neutralizing antibody. Surprisingly, TGF-β neutralization enhanced the migration of *SPARC*$^{-/-}$ cells only (Fig. 3 B and Fig. S2 A). The ALK5 inhibitor SB431542 had a similar effect, suggesting that TGF-β receptor activity is enhanced in the absence of SPARC expression (Fig. 3 C). We next attempted to recapitulate our findings using 10T1/2 cells. We found that shRNA-mediated knockdown of SPARC impaired 10T1/2 cell migration in a TGF-β–dependent manner (Fig. 3 D and Fig. S2 B). Furthermore, neutralization of SPARC using a monoclonal antibody reduced 10T1/2 cell migration in a TGF-β–dependent manner (Fig. 3 E). Thus, SPARC prevents TGF-β–dependent attenuation of pericyte migration.

We found that TGF-β1 could reduce *SPARC*$^{+/+}$ pericyte and 10T1/2 cell migration and, therefore, hypothesized that increased TGF-β1 expression in *SPARC*$^{-/-}$ pericytes was responsible for their migration phenotype. Surprisingly, analysis of TGF-β1 in cell lysates and conditioned media revealed no differences in TGF-β1 levels in *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes (Fig. 4 A). RT-PCR also revealed no difference in TGF-β1 expression between *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes (Fig. 4 B). However, we did observe an increase in *PAI1* expression in *SPARC*$^{-/-}$ pericytes, which is a canonical TGF-β1 response gene (Fig. 4 B). Using quantitative PCR (qPCR), we confirmed that SPARC reduced the expression of canonical TGF-β1 response genes in pericytes (Fig. 4 C). Interestingly, *SPARC*$^{-/-}$ pericytes seemed to be more sensitive to exogenous TGF-β1 (Fig. S3 B). As TGF-β1 signaling is initiated at the cell surface, we hypothesized that *SPARC*$^{-/-}$ pericytes had more surface-associated TGF-β1. Indeed, we observed more TGF-β1 on the surfaces of nonpermeabilized *SPARC*$^{-/-}$ pericytes (Fig. S3 A). We also assessed the level of surface-associated TGF-β1 using an impermeable cross-linker. We found that *SPARC*$^{-/-}$ pericytes had more surface-associated TGF-β1 compared with *SPARC*$^{+/+}$ cells, a feature that was reversed by recombinant SPARC (Fig. 4 E). We next tested the possibility that *SPARC*$^{-/-}$ pericytes were responding to endogenous TGF-β1. For this, we examined phosphorylated SMAD2 (pSMAD2) in response to shRNA-mediated knockdown of TGF-β1. We found that knockdown of TGF-β1 reduced SMAD2 phosphorylation only in *SPARC*$^{-/-}$ pericytes but did not inhibit their capacity to respond to exogenous TGF-β1 (Fig. S3 C and Fig. 4 D). Finally, using TGF-β1 knockdown, we found that endogenous TGF-β1 was required for the migration phenotype of *SPARC*$^{-/-}$ pericytes (Fig. 4 F). Thus, SPARC reduces activation of endogenous TGF-β1.

SPARC interacts with the TGF-β accessory receptor endoglin

SPARC is a secreted glycoprotein; therefore, we hypothesized that control of TGF-β1 activity may be mediated through interactions with TGF-β1 receptors. SPARC can interact with a soluble form of TβRII but only in the presence of recombinant TGF-β1 (Francki et al., 2004). TGF-β1 binds sequentially to its receptors: active TGF-β1 first binds to TβRII, which can be in a heteromeric complex with endoglin, which then recruits a type I receptor, such as ALK5. We immunoprecipitated each of these receptors from 10T1/2 cells and found that SPARC specifically coprecipitated with endoglin (Fig. 5 B). SPARC was also detected in endoglin immune complexes from *SPARC*$^{+/+}$ pericytes (Fig. 5 B, right). We confirmed this interaction using solid-phase binding assays (Fig. 5 C). Furthermore, immunofluorescent staining of primary *SPARC*$^{+/+}$ pericytes revealed that SPARC and endoglin associate in distinct punctate structures (Fig. 5 A). This interaction links SPARC to TGF-β1 signaling.

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

The function of endoglin in TGF-β1 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 (Gougos et al., 1992; Conley et al., 2004; Koleva et al., 2006;

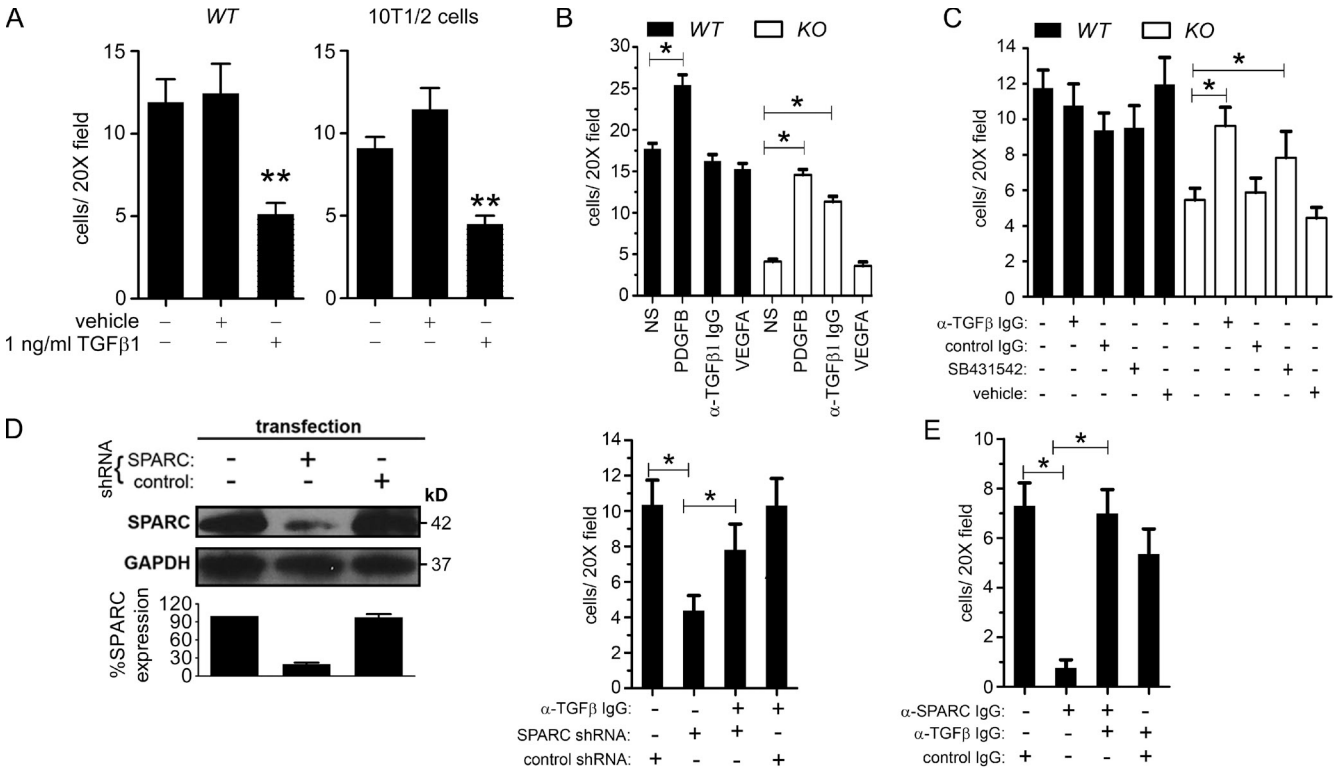

Figure 3. **TGF-β limits transwell migration of pericytes in the absence of SPARC.** (A) TGF-β1 reduces *SPARC*$^{+/+}$ pericyte and 10T1/2 cell migration. Cells were allowed to migrate in the presence of vehicle or 1 ng/ml TGF-β1. Error bars represent SEM (**, P < 0.001 vs. vehicle). (B) Neutralization of TGF-β enhances *SPARC*$^{-/-}$, but not *SPARC*$^{+/+}$, pericyte migration. Cell migration in the presence of no stimulation (NS), 10 nM PDGF-BB, 25 ng/ml anti–TGF-β IgG (α–TGF-β IgG), or 50 ng/ml VEGF-A was assessed. (C) Inhibition of ALK5 enhances *SPARC*$^{-/-}$ pericyte migration. The effect of 25 ng/ml α–TGF-β or control IgG, 10 µM ALK5 inhibitor (SB431542), or ALK5 inhibitor vehicle alone (vehicle) on the migration of primary pericytes is shown. (D) Knockdown of SPARC in 10T1/2 cells impairs migration in a TGF-β–dependent manner. 10T1/2 cells were transfected with SPARC or control shRNA for 48 h and then used in a transwell assay. Western blot to confirm knockdown is presented on the left. Cells were allowed to migrate in the presence of 25 ng/ml α–TGF-β IgG where indicated. (E) Anti-SPARC IgG reduces 10T1/2 cell migration in a TGF-β–dependent manner. 10T1/2 cells were allowed to migrate in the presence of 25 ng/ml control IgG, 25 ng/ml anti-SPARC IgG (clone 293; α-SPARC IgG), 25 ng/ml α–TGF-β IgG plus 25 ng/ml α-SPARC IgG, or 25 ng/ml α–TGF-β IgG plus 25 ng/ml control IgG for 6 h. All experiments were performed in triplicate at least twice with similar results. Mean values are presented. Error bars represent SEM (*, P < 0.05). *WT*, *SPARC*$^{+/+}$; *KO*, *SPARC*$^{-/-}$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Lee et al., 2008b). Therefore, we explored whether endoglin exhibited differences in localization and activity in SPARC-deficient pericytes. We first assessed endoglin association with focal complexes of spreading *SPARC*$^{+/+}$ or *SPARC*$^{-/-}$ pericytes. Vinculin-rich focal complexes were observed in spreading *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes (Fig. 6 A). We found that endoglin localized with focal complexes only in *SPARC*$^{-/-}$ pericytes (Fig. 6 A, insets and magnified images). To determine whether endoglin aberrantly associates with focal adhesion machinery in the absence of focal complexes, we immunoprecipitated endoglin complexes from *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes in suspension or seeded on plastic or fibronectin and probed for FAK, as FAK becomes incorporated into maturing focal complexes (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. 6 B). We confirmed that FAK-associated endoglin was SPARC dependent using recombinant SPARC (Fig. 6 C). These results suggest that SPARC prevents endoglin from incorporating into focal complexes.

Next, we asked whether endoglin was required for elevated basal SMAD2 phosphorylation in *SPARC*$^{-/-}$ pericytes. We found shRNA-mediated knockdown of endoglin resulted in decreased SMAD2 phosphorylation in *SPARC*$^{-/-}$, but not *SPARC*$^{+/+}$, pericytes (Fig. 6 D).

We then asked whether endoglin participates in the migration phenotype of *SPARC*$^{-/-}$ pericytes. To test this, we used endoglin shRNA in 10T1/2 cells while targeting SPARC with either shRNA or a monoclonal antibody in a transwell assay. Endoglin knockdown had no effect on 10T1/2 cells when SPARC was not manipulated (e.g., control shRNA or control antibody; Fig. 6, E and F). However, knockdown of endoglin increased migration of cells transfected with SPARC shRNA or treated with an anti-SPARC antibody. Collectively, these results suggest focal complex–associated endoglin facilitates TGF-β1 activity and that SPARC functions to limit this process.

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

Endoglin does not directly activate TGF-β1–mediated processes (Koleva et al., 2006; Lee et al., 2008b). Association of endoglin with focal complexes and the regulation of migration and SMAD2 phosphorylation in *SPARC*$^{-/-}$, but not *SPARC*$^{+/+}$, pericytes suggested that, perhaps, in the absence of SPARC, endoglin was

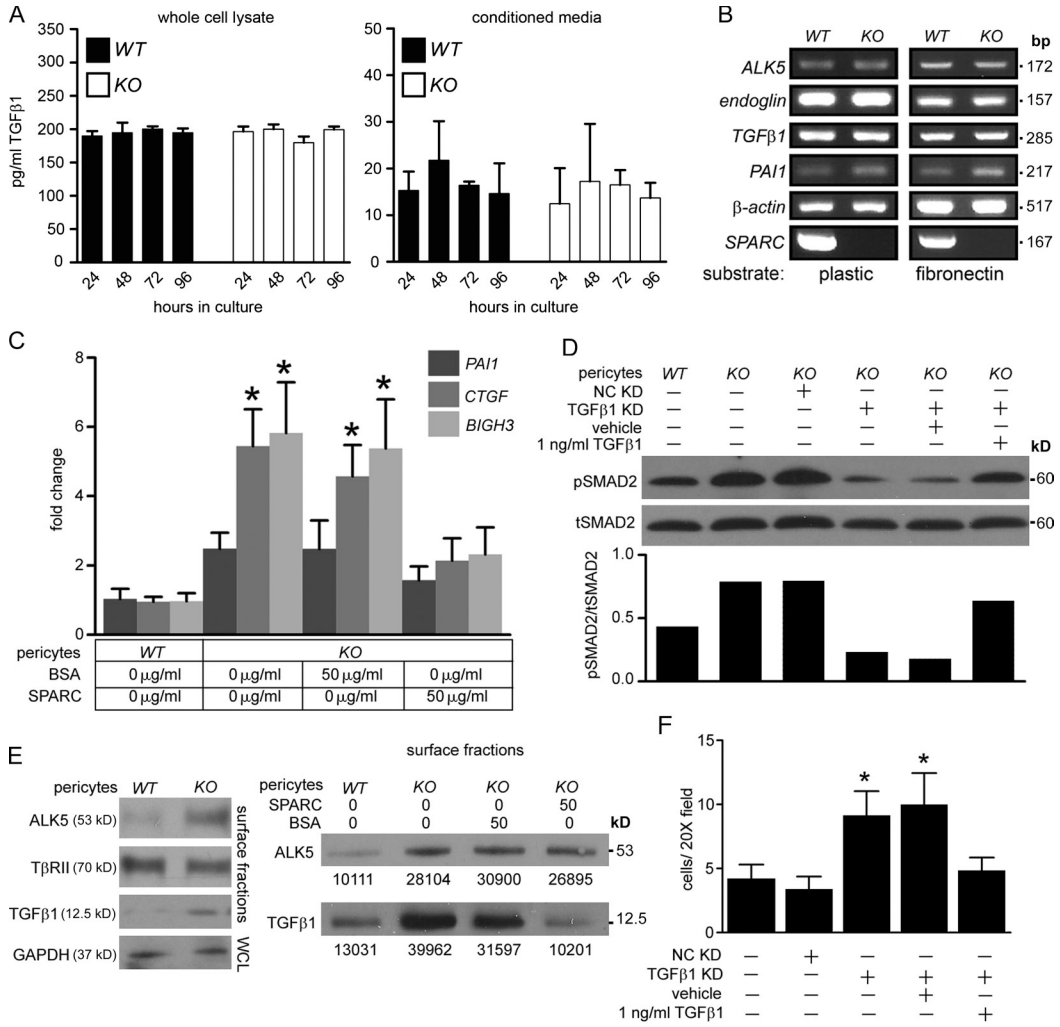

Figure 4. ***SPARC*$^{-/-}$ pericytes exhibit increased basal TGF-β activity.** (A) *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes express similar levels of TGF-β1 protein. Cells lysates and conditioned media were harvested after culture for the indicated times. TGF-β1 concentration was detected using a TGF-β1 ELISA. (B) Primary *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes express similar levels of TGF-β1 message. RT-PCR for the indicated genes was performed with cDNA harvested from cells cultured on either plastic or fibronectin. (C) *SPARC*$^{-/-}$ pericytes exhibit higher canonical TGF-β1 response gene expression. qPCR was performed on cells treated as indicated. Error bars represent SEM (*, $P < 0.05$ vs. WT). (D) Endogenous TGF-β1 induces SMAD2 phosphorylation in *SPARC*$^{-/-}$ pericytes. Cells were transfected with either negative control (NC) or TGF-β1 shRNA where indicated 48 h before preparing lysates. Cells treated with TGF-β1 received treatment 5 h before cell lysis. Lysates were probed for tSMAD2 and pSMAD2 by SDS-PAGE and Western blotting. pSMAD2 levels were normalized with ImageJ software. (E) *SPARC*$^{-/-}$ pericytes retain more surface TGF-β1 than *SPARC*$^{+/+}$ counterparts. Surface proteins were labeled, purified, and subjected to SDS-PAGE and probed for the indicated proteins by Western blotting (left). Recombinant SPARC decreases TGF-β1 levels on the surface of *SPARC*$^{-/-}$ pericytes (right). Cells were cultured as in C before surface protein extraction and Western blot analysis. Pixel area under the curve was generated using ImageJ software, and these values are presented under their respective bands. (F) Knockdown (KD) of TGF-β1 enhances migration of *SPARC*$^{-/-}$ pericytes. Cells were transfected with the indicated shRNA for 48 h before use in the transwell assay. Cells were treated with TGF-β1 for the duration of the assay only. All experiments were performed at least twice with identical results. Mean values are presented. Error bars represent SEM (*, $P < 0.05$). *WT*, *SPARC*$^{+/+}$; *KO*, *SPARC*$^{-/-}$; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; WCL, whole-cell lysate.

cooperating with factors that positively regulate TGF-β signaling. αV integrins facilitate cell adhesion and migration and can directly activate latent TGF-β1 (Delannet et al., 1994; Klemke et al., 1994; Liaw et al., 1995; Stefansson and Lawrence, 1996). Therefore, we sought to determine whether endoglin cooperated with αV integrins to enhance TGF-β1 activity in *SPARC*$^{-/-}$ pericytes. We found that *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes express similar levels of αV integrins by RT-PCR and flow cytometry (Fig. 7 A). Furthermore, *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes express β integrin subunits that have been implicated to participate with αV integrin in mediating TGF-β1 activation (Munger et al., 1998; Mu et al., 2002; Ludbrook et al., 2003; Annes et al., 2004). We also found no differences in αV integrin–mediated migration between *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ pericytes (Fig. 7 B). We then asked whether endoglin-associated αV integrins are required for the increased basal TGF-β1 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. 7 C). We then examined whether αV integrin was present in endoglin immune complexes. αV integrin was detected in *SPARC*$^{-/-}$ pericyte immune complexes regardless of whether cells were adherent or in suspension (Fig. 7 D). αV integrin was also detected in *SPARC*$^{+/+}$ pericyte

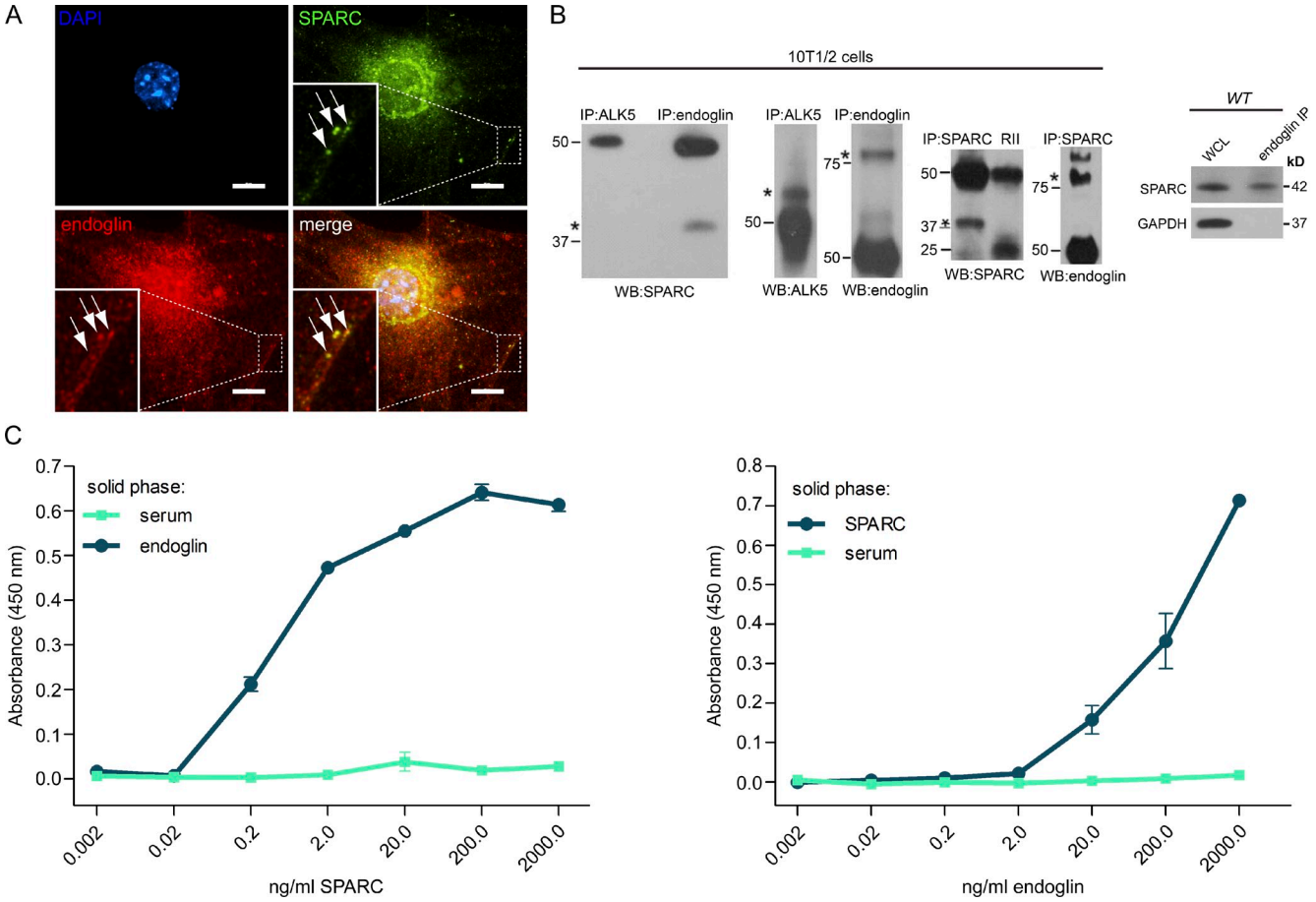

Figure 5. **SPARC interacts with TGF-β1 accessory receptor endoglin.** (A) SPARC and endoglin colocalize in primary *SPARC*$^{+/+}$ pericytes. Pericytes adhered to silane-coated slides were subjected to immunofluorescence with anti-SPARC IgG and antiendoglin IgG. DAPI was used to visualize nuclei. Inset shows colocalization. Arrows indicate punctuate areas of colocalization. Bars, 10 µm. (B) SPARC coprecipitates with endoglin immune complexes. 10T1/2 cell lysates were incubated anti-ALK5, antiendoglin, anti-SPARC, or anti-TβRII (RII) IgGs. IgG-bound complexes were precipitated with protein A/G agarose beads. The indicated complexes were subjected to SDS-PAGE and probed for the indicated proteins by Western blotting. Asterisks indicate the target protein. Molecular masses in kilodaltons are labeled. IgG V_H corresponds to the 50-kD band. IP, immunoprecipitation target; WB, Western blot target. SPARC was also detected in endoglin immune complexes from *SPARC*$^{+/+}$ pericytes (*WT*). (C) SPARC interacts with endoglin in solid-phase binding assays. 96-well plates were coated with either 5 µg/ml endoglin (left), 5 µg/ml SPARC (right), or control serum. Soluble SPARC or endoglin was added to the indicated plate in triplicate at increasing concentrations and detected using anti-SPARC or antiendoglin IgG. All experiments were performed at least three times with identical results. Binding is expressed as mean absorbance. Errors bars represent SEM. Epifluorescent images are presented in A. WCL, whole-cell lysate.

immune complexes, albeit at lower levels (Fig. 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 and endoglin association. Thus, we assessed the effect of adding recombinant SPARC to *SPARC*$^{-/-}$ pericytes on the amount of endoglin-associated αV integrin. We found that exogenous SPARC reduced endoglin-associated αV integrin while having no effect on total αV integrin levels (Fig. 7 E). Collectively, these results show that SPARC, by blocking endoglin from interacting with αV integrins, can reduce TGF-β activity.

Discussion

SPARC reduces the capacity of pericytes to perceive TGF-β1

In the current study, we demonstrate that SPARC promotes pericyte migration by reducing TGF-β1–induced responses. To perform this function, SPARC interacts with the TGF-β1 accessory receptor endoglin. In the absence of SPARC, endoglin associates with αV integrins and enhances TGF-β1 signaling to impair pericyte migration.

During angiogenesis, pericytes migrate to nascent vessels where TGF-β1 signaling impedes further migration and triggers pericyte-induced vessel quiescence (Courtoy and Boyles, 1983; Antonelli-Orlidge et al., 1989; Sato and Rifkin, 1989; Stefansson and Lawrence, 1996; Hirschi et al., 1998; Darland and D'Amore, 2001). As TGF-β1 is present in the extracellular milieu throughout endothelial tube formation, the capacity of pericytes to respond to TGF-β1 must be regulated spatially. Mechanisms that contribute to such regulation remain unknown. We found that SPARC was expressed in pericytes during vascular morphogenesis in PDAC, and pericyte recruitment was reduced in the absence of SPARC. We propose that SPARC can block the capacity of pericytes to respond to TGF-β1 during angiogenesis and, thus, facilitate cell migration to nascent blood vessels, based on the following observations: First, SPARC-deficient

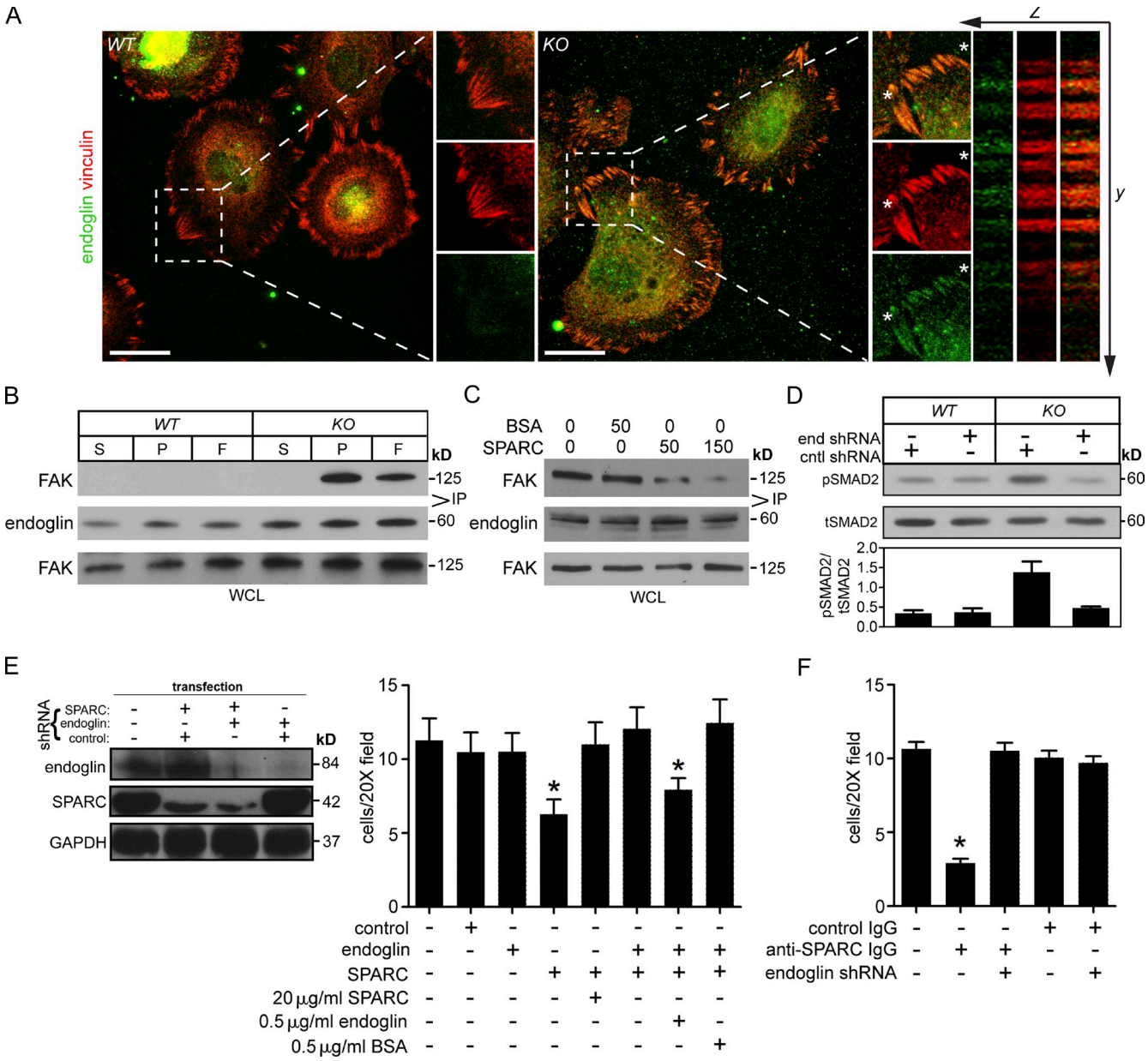

Figure 6. **Endoglin associates with focal complexes in *SPARC*$^{-/-}$ pericytes.** (A) Endoglin colocalizes with vinculin plaques in *SPARC*$^{-/-}$ pericytes. Cells were plated onto fibronectin-coated slides for 120 min. Cells were fixed and stained with antivinculin and antiendoglin IgG and visualized using a confocal microscope (TCS SP5; maximum intensity projections from 16–0.15-µm z stacks per stain were generated using ImageJ software and are presented). ZY planes are presented from the regions between the asterisks in split channel images. Bars, 20 µm. (B) FAK coprecipitates with endoglin immune complexes from *SPARC*$^{-/-}$ pericytes. Endoglin was immunoprecipitated from lysates harvested from cells in suspension (S) or adhered to plastic (P) or fibronectin (F). Complexes were then probed for FAK or endoglin by SDS-PAGE and Western blotting. WCL, whole-cell lysates. (C) Recombinant SPARC reduces FAK-associated endoglin in *SPARC*$^{-/-}$ pericytes. *SPARC*$^{-/-}$ pericytes were incubated with either BSA or recombinant SPARC at 0, 50, or 150 µg/ml for 6 h. Endoglin was immunoprecipitated from cell lysates, and complexes were subjected to SDS-PAGE and Western blotting for endoglin and FAK. (D) Knockdown of endoglin reduces SMAD2 phosphorylation in *SPARC*$^{-/-}$ pericytes. Pericytes were transfected with endoglin or control shRNA for 48 h. Lysates were prepared and subjected to SDS-PAGE. tSMAD2 and pSMAD2 were detected by Western blotting. pSMAD2 levels were normalized with ImageJ software. (E) Knockdown of endoglin reverses the effect of silencing SPARC on 10T1/2 cell transwell migration. Cells were transfected with the indicated shRNA for 48 h and then allowed to migrate in the indicated conditions. (F) Knockdown of endoglin reverses the effect of neutralizing SPARC on 10T1/2 cell transwell migration. Cells were transfected as in E and allowed to migrate in the presence or absence of 25 ng/ml anti-SPARC or control IgG as indicated. All experiments were performed at least twice with identical results. Mean values are presented. Error bars represent SEM (*, $P < 0.05$). *WT*, *SPARC*$^{+/+}$; *KO*, *SPARC*$^{-/-}$. Leica confocal images are presented in A (see Materials and methods). GAPDH, glyceraldehyde 3-phosphate dehydrogenase; cntl, control; end, endoglin.

pericytes migrated less in vitro, an effect that was reversed when adding back recombinant SPARC, blocking TGF-β–TGF-β receptor ligation, inhibiting ALK5 kinase activity, or knocking down TGF-β1. Second, SPARC-deficient pericytes exhibited increased basal TGF-β1–induced SMAD2 phosphorylation and activity. Third, SPARC deficiency resulted in increased TGF-β1 associated with pericyte surfaces while not effecting levels of TGF-β1 mRNA, cytosolic TGF-β1, or secreted TGF-β1.

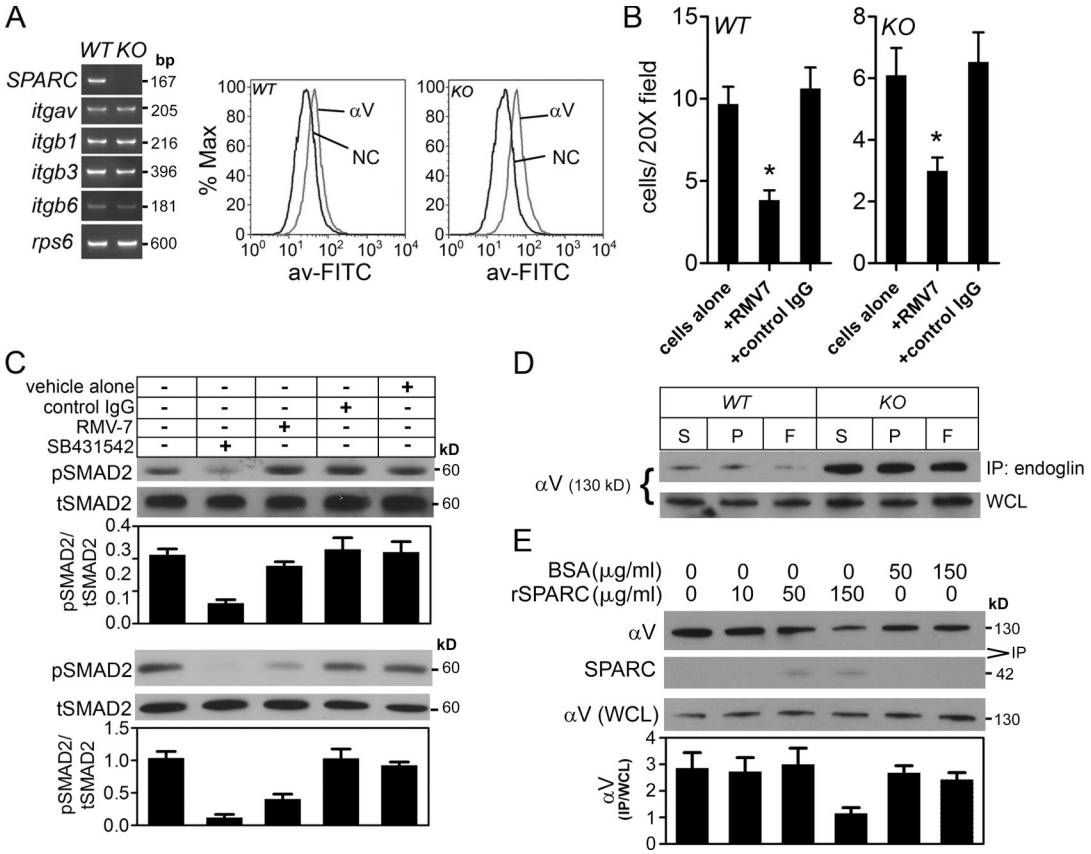

Figure 7. **αV integrin interacts with endoglin and mediates TGF-β activity in primary *SPARC*$^{-/-}$ pericytes.** (A) Integrin expression profile of primary pericytes. RT-PCR detection of SPARC, αV integrin (*itgav*), β1 integrin (*itgb1*), β3 integrin (*itgb3*), β6 integrin (*itgb6*), and RPS6 (*rps6*). (right) 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 μg/ml. Control cells were stained with secondary alone. av, avidin; NC, negative control. (B) αV integrins regulate transwell migration of primary pericytes. Pericytes were allowed to migrate in the presence of 20 μg/ml RMV-7 or 20 μg/ml control IgG. Mean values are presented. Error bars represent SEM (*, P <0.05). (C) αV integrins regulate TGF-β activity in primary *SPARC*$^{-/-}$ pericytes. Primary *SPARC*$^{+/+}$ or *SPARC*$^{-/-}$ pericytes were incubated overnight in 1.5% serum in the presence of 10 μM SB431542, 20 μg/ml RMV-7, 20 μg/ml control IgG, or vehicle alone as indicated. pSMAD2 and tSMAD2 levels were determined by SDS-PAGE and Western blotting. pSMAD2 levels were normalized to tSMAD2 using ImageJ software. (D) αV integrins associate with endoglin in *SPARC*$^{-/-}$ pericytes. Endoglin was immunoprecipitated from lysates harvested from cells in suspension (S) or adhered to plastic (P) or fibronectin (F). Complexes were then subjected to SDS-PAGE and probed for αV integrin by Western blotting. (E) SPARC blocks the αV integrin–endoglin interaction. *SPARC*$^{-/-}$ pericytes were incubated with either BSA or recombinant SPARC (rSPARC) at the indicated concentrations for 6 h. Endoglin was immunoprecipitated from cell lysates, and complexes were subjected to SDS-PAGE. αV integrin and SPARC levels were determined by Western blotting. Coprecipitating αV integrin was normalized to αV integrin in whole-cell lysates (WCL) using ImageJ software. IP, immunoprecipitation. All experiments were repeated at least twice with the same results. *WT*, *SPARC*$^{+/+}$; *KO*, *SPARC*$^{-/-}$.

SPARC thus may control TGF-β1 perception by pericytes during blood vessel formation. Interestingly, we found that *SPARC*$^{-/-}$ pericytes were more sensitive to exogenous TGF-β1 (Fig. S3 B). Integrins αVβ3 and αVβ5 have been shown to promote TGF-β1 signaling and target gene expression by enhancing TGF-β receptor activity through direct receptor interactions; therefore, it seems reasonable that endoglin–αV integrin complexes have an amplified response to exogenous TGF-β1 (Scaffidi et al., 2004; Asano et al., 2006a,b; Galliher and Schiemann, 2006).

In spite of our findings, the precise mechanism through which SPARC regulates TGF-β1 activity is still unclear. This is because of several independent studies with opposing conclusions on the effect of SPARC on TGF-β1 activity (Schiemann et al., 2003; Francki et al., 2004; Chlenski et al., 2007). In the current study, we found that SPARC required endoglin to regulate TGF-β1 activity in pericytes, a finding that suggests its effects may be dependent on this accessory receptor in other cells types. Studies assessing the contribution of TGF-β1 accessory receptors to SPARC activity will undoubtedly shed light on why SPARC and TGF-β1 expression temporally overlap during development and disease.

SPARC controls pericyte migration by regulating endoglin function

Endoglin is a critical component of the TGF-β–signaling machinery and is required for development of the vasculature. Genetic ablation of endoglin in mice results in embryonic lethality from defective vascular remodeling, a phenotype that resembles that of TGF-β1 KO mice (Dickson et al., 1995; Bourdeau et al., 1999; Li et al., 1999; Arthur et al., 2000). Interestingly, endoglin is not required for formation of the initial vascular plexus,

rather vessel defects result from lack of mural cell recruitment to the early vascular network. Vascular cell expression of endoglin is increased during angiogenesis; however, the function of endoglin in pericytes has been unclear. Similarly, αV integrin is expressed by mural cells, but its function on these cells during angiogenesis is unclear (Stawowy et al., 2003; Gao and Brigstock, 2004). Our results thus place these proteins together with SPARC in a mechanism to regulate the activation of latent TGF-β1. We found that endoglin was required for the enhancement of TGF-β–induced responses we observed in the absence of SPARC. SPARC also prevented endoglin from associating with αV integrins, likely via a mechanism involving direct interaction with the extracellular domain of endoglin. We propose that the capacity of endoglin to block pericyte migration in the absence of SPARC requires its association with focal complex–associated proteins. In support of this, we found that recombinant SPARC disrupted endoglin–αV integrin and endoglin–FAK complex formation, with disruption of endoglin–FAK complexes by SPARC being more efficient. Other groups have shown that FAK and other focal adhesion proteins dissociate from integrins after internalization (Finnemann, 2003; Pellinen and Ivaska, 2006; Thomas et al., 2010). We found that endoglin–αV integrin complexes were not dependent on the formation of focal adhesions, a finding that suggests these complexes persist upon integrin internalization. Such a scenario would result in a pool of endoglin–αV complexes that were not associated with focal adhesions and might be less sensitive to SPARC-induced dissociation. These complexes would likely make it difficult to discern complex dissociation at focal adhesions in our assay. Thus, we would predict that relatively high concentrations of SPARC would be required to perturb endoglin–αV complexes (Fig. 7 E). Conversely, we found endoglin–FAK complexes only when *SPARC*$^{-/-}$ pericytes were allowed to form focal adhesions. This suggests that prevention of endoglin from interacting with focal adhesion proteins (e.g., with SPARC) would result in the disruption of endoglin–FAK complexes. Indeed, we saw endoglin–FAK complex dissociation at a threefold lower concentration of SPARC compared with endoglin–αV complexes.

Endoglin can control cell migration, at least in part, via intracellular interactions. Previous studies have demonstrated direct interaction between the cytoplasmic PDZ-interacting motif of endoglin and GIPC1. This interaction resulted in retention of surface-associated endoglin in focal complexes, increased SMAD phosphorylation, and reduced cell migration (Lee et al., 2010; Ray et al., 2010). The cytoplasmic domain of endoglin also facilitates blockade of zyxin and ZRP-1 recruitment into maturing focal complexes; thus, endoglin may reduce migration as a result of suboptimal focal adhesion assembly (Conley et al., 2004; Sanz-Rodriguez et al., 2004). In addition to intracellular interactions, our results demonstrate that endoglin can influence cell migration via extracellular interactions. Determining whether the effect of SPARC on endoglin activity requires the cytoplasmic tail of endoglin and subsequent changes in focal complex assembly will provide insight on the mechanism of SPARC control of pericyte behavior and may help explain how SPARC regulates cell morphology in general.

Activated TGF-β receptors regulate pericyte migration via their inherent kinase activity; for example, TGF-β1 inhibits migration by stimulating ALK5-dependent phosphorylation of SMADs and p38 (Feinberg et al., 2004). TGF-β1 signaling is potentiated upon recruitment of TGF-β receptors into focal complexes, though the mechanisms that drive receptor recruitment to these structures are unclear (Scaffidi et al., 2004; Asano et al., 2006b; Galliher and Schiemann, 2006; Lee et al., 2010). In addition to interacting with intracellular components of focal adhesions, endoglin also interacts with TβRII in the absence of a bound ligand, a feature that suggests it may serve to bridge the gap between TGF-β1 signaling and focal complexes (Barbara et al., 1999; Guerrero-Esteo et al., 2002). We found that in the absence of SPARC, endoglin facilitated increased SMAD2 phosphorylation. As endoglin does not exhibit kinase activity, this was likely the result of TGF-β receptor recruitment to focal complexes.

TβRII/ALK5 signaling is required for the decreased migration observed in the absence of SPARC; however, it remains to be determined whether endoglin localization to focal complexes is dependent on TβRII/ALK5 activity. In our model, endoglin links these receptors to TGF-β1, which predicts that association of endoglin with focal complexes is upstream of any kinase activity.

Endoglin links TGF-β receptors to αV integrin complexes

The question then becomes: how does focal complex–associated endoglin enhance TGF-β1 activity? We propose that endoglin bridges TGF-β receptors and αV integrin–associated TGF-β1. Our results support this proposal based on the following: First, αV integrins were found in the endoglin-enriched focal complexes observed in the absence of SPARC. Second, recombinant SPARC increased migration of *SPARC*$^{-/-}$ pericytes while decreasing endoglin–αV integrin complex formation. Third, αV integrins enhanced ALK5/TβRII activity but only in the absence of SPARC. These results are in line with other studies demonstrating that SPARC can interact with and regulate focal adhesion proteins (Murphy-Ullrich et al., 1995; Motamed and Sage, 1998; Shi et al., 2004; Barker et al., 2005; Weaver et al., 2006, 2008; Nie et al., 2008). Recently, SPARC was shown to interact with β1 integrins (Nie et al., 2008; Weaver et al., 2008). Our data do not rule out the possibility that SPARC interacts with endoglin and β integrins to control αV integrin–TGF-β1 signaling. Indeed, αVβ1 integrin can bind to the latency-associated peptide (LAP), though actual activation of latent TGF-β1 has yet to be demonstrated clearly (Munger et al., 1998).

TGF-β1 LAP contains an RGD domain to which all five αV integrins can bind (Munger et al., 1999; Mu et al., 2002; Ludbrook et al., 2003). Furthermore, interaction of latent TGF-β1 with αVβ3, αVβ5, αVβ6, and αVβ8 results in the presentation of TGF-β1 to its receptors. In our model, in the absence of SPARC, αV integrins interact with latent TGF-β1 and present the active protein to endoglin-associated TGF-β receptors, facilitating the inhibition of migration. In wild-type (WT) cells, SPARC interacts with endoglin and prevents it from recruiting TGF-β receptors to αV integrin–latent TGF-β1 complexes, promoting pericyte migration (Fig. 8).

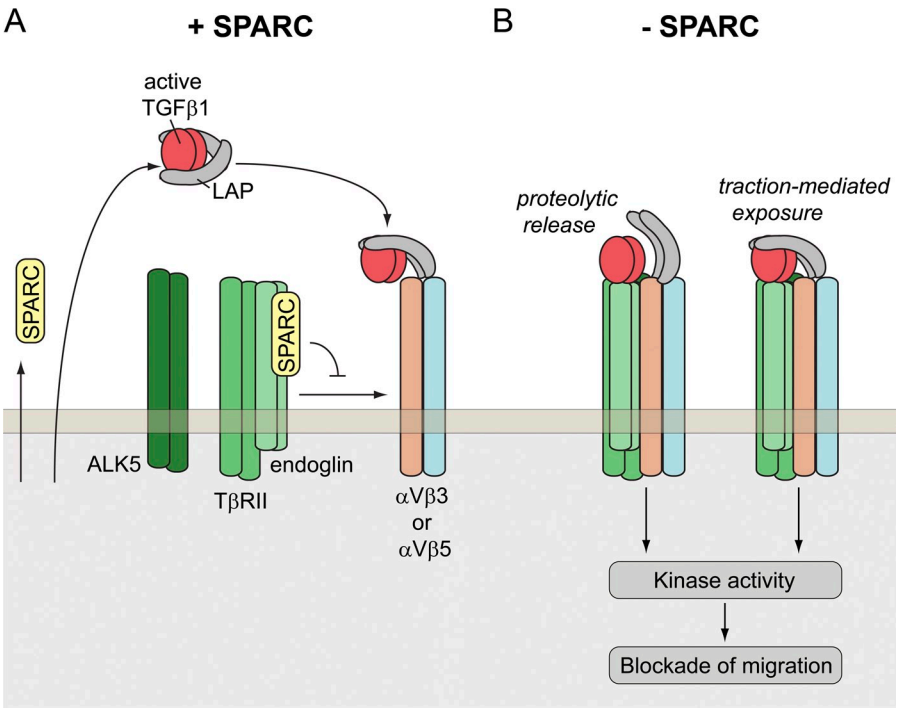

Figure 8. **Proposed model of how SPARC regulates pericyte migration through endoglin and TGF-β1.** (A) TGF-β1 is secreted as a latent protein associated with LAP and cannot bind TGF-β receptors. LAP binds αV integrins via its RGD motif. SPARC binds endoglin, blocking its association with αV integrins, thereby promoting pericyte migration. (B) In the absence of SPARC, endoglin bridges TβRII/ALK5 with αV integrin–associated active TGF-β1 and promotes signals that blocks pericyte migration. This model predicts that as pericytes come into contact with nascent blood vessels, SPARC is somehow removed from the receptor complex, allowing TGF-β receptors to engage αV integrin–bound TGF-β1. During angiogenesis, SPARC undergoes proteolysis in a regulated fashion (Lane et al., 1994). This proteolysis produces SPARC fragments with various activities and, thus, may provide a mechanism for controlling SPARC–endoglin interactions.

There are currently two known mechanisms of αV integrin–mediated activation of TGF-β1, one requiring protease activity and the other being protease independent. Protease-independent activation of latent TGF-β1 has been shown to occur with αVβ3, αVβ5, and αVβ6 integrins (Ludbrook et al., 2003; Annes et al., 2004; Wipff et al., 2007). In this mechanism, traction is proposed to induce the release of active TGF-β1 from the latent complex. Binding of active TGF-β1 requires TGF-β receptors to be in close proximity, a feature that explains why release of active TGF-β1 into the culture media is never observed. Interestingly, we failed to detect changes in active TGF-β1 in culture media, suggesting this mechanism may be involved.

Protease-dependent activation of TGF-β1 has only been demonstrated with αVβ8: αVβ8 binds latent TGFβ1 and recruits MT1-MMP, which then releases active TGF-β1 via proteolytic cleavage of LAP (Mu et al., 2002). As we failed to detect changes in TGF-β1 in culture media, it is unlikely that an αVβ8/MT1-MMP axis is involved in SPARC-mediated regulation of pericyte behavior.

Conclusion

Endoglin is an established regulator of endothelial cell behavior. We describe here a novel mechanism in which endoglin cooperates with SPARC to regulate pericyte responses to TGF-β1. Our findings suggest that during angiogenesis, SPARC functions to restrict pericyte perception of TGF-β1 in the angiogenic milieu through its interaction with endoglin. Such control is critical, as TGF-β1 is present early in the angiogenic cascade, yet TGF-β1 signaling in pericytes must occur after formation of endothelial tubes.

SPARC was expressed in both resting and mobilized pericytes, an observation that suggests the function of SPARC is also controlled. Indeed, previous studies have demonstrated that SPARC can be cleaved by plasmin, whose own expression is temporally regulated throughout angiogenesis (Lane et al., 1992, 1994; Iruela-Arispe et al., 1995). Furthermore, the integrin profile of pericytes changes during angiogenesis, a phenomenon that may also affect SPARC-mediated blockade of TGF-β1. Experiments addressing how these events regulate SPARC activity will undoubtedly yield important insights on how matricellular proteins, such as SPARC, regulate critical physiological processes.

Materials and methods

Animal husbandry

PDAC (*P48Cre*$^{+}$*:LSLKrasG12D*$^{+}$*:INK4A*$^{lox/lox}$) mice were crossed with *SPARC*$^{+/+}$ or *SPARC*$^{-/-}$ mice to produce WT *PDAC* or *SPARC-null PDAC* (knockout [KO] *PDAC*) mice. For tumor analyses, mice were sacrificed once becoming moribund, with at least six mice per group. Tumors were preserved in formalin or snap frozen using liquid nitrogen. Animal experiments were performed at the University of Texas Southwestern Medical Center at Dallas in compliance with the Animal Welfare Act, the Public Health Service Policy, and the U.S. Government Principles Regarding the Care and Use of Animals.

Antibodies

Antibodies to the following proteins were used for indirect immunofluorescent microscopy: α-SMA (NeoMarkers), NG2 (AB5320; Millipore), desmin (Ab907; Millipore), vinculin (V4139; Sigma-Aldrich), MECA32, SPARC (R&D Systems), total TGF-β (SC146; Santa Cruz Biotechnology, Inc.), and endoglin (MJ7/18). For function-blocking assays, a pan–TGF-β–neutralizing antibody was purchased from R&D Systems (1D11), an αVβ6-blocking antibody was a gift from D. Sheppard (University of California, San Francisco, San Francisco, CA), and an αV integrin–blocking antibody was purchased from BioLegend (RMV-7). The hybridomas that produce mAb293 and mAb303 were grown in our laboratory and purified by protein A chromatography. For Western blots, ALK5 (SC-398; Santa Cruz Biotechnology, Inc.), TβRII (SC-220; Santa Cruz Biotechnology, Inc.), endoglin (clone MJ7/18), αV integrin (AB1930; Millipore), FAK (3285; Cell Signaling Technology), phospho-SMAD2 (AB3849 serine 465/467; Millipore), and total SMAD2 (tSMAD2; 3107; Cell Signaling Technology) were used. For solid-phase binding assays, endoglin (MJ7/18) and SPARC (mAb 236)

were used. For immunoprecipitations, SPARC (mAb303), ALK5, TβRII (SC-220), and endoglin (MJ7/18) were used. The hybridomas MECA32 and MJ7/18, developed by E.C. Butcher (Stanford University, Palo Alto, CA), were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development, and maintained by The University of Iowa.

Primary pericyte isolation, cell culture, and transfections

Mouse pancreata from 4-wk-old *SPARC*$^{+/+}$ and *SPARC*$^{-/-}$ mice were minced and then subjected to digestion with 1% collagenase type 1, DME, 10 mM Hepes, 1% fetal bovine serum, and PBS at 37°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-µm 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 and then washed three times in wash buffer to remove NaN_2. Bead-bound cells were separated from unbound cells using a cell separation magnet (IMagnet; BD). Primary pericytes were maintained in 10% fetal bovine serum–supplemented DME 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 DME. Primary pericytes were transfected using Lipofectamine 2000 (Invitrogen), whereas 10T1/2 cells were transfected using Fugene (Roche). For shRNA knockdown of SPARC, endoglin, and TGF-β1, shRNA expression plasmids were purchased from Sigma-Aldrich (MISSION). 2 µg plasmid DNA was mixed with 3 µl transfection reagent and used to transfect 150,000 cells. Cells were used 48 h after transfection. For negative control transfections, a nontargeting shRNA expression plasmid was used (MISSION).

Cord formation assay

10,000 bEnd.3 endothelial cells were plated onto matrigel-coated 3-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 h in DME 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-Aldrich). Experiments were performed three times and in triplicate. Images were taken at a 4× 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 NIS-Elements software (Nikon). For cord width measurements, widths were taken halfway into the length of each cord.

Transwell assay

Transwell inserts with 8-µm 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 µl of 1-µg/µl fibronectin for 1 h at 37°C. Inserts were then used immediately for experiments (Sigma-Aldrich). 7,000 or 5,000 primary pericytes or 10T1/2 cells were added into the insert reservoir in DME in a total volume of 125 µl, whereas DME 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 h. Cells on the noncoated 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 performed in triplicate and repeated two or three times as indicated in the figure legends.

TGF-β ELISA and TGF-β1 response gene expression

A TGF-β1 ELISA kit (TGF-β1 EMAX Immunoassay G7591) that detects the active form of TGF-β1 was purchased from Promega. Sample preparation for active TGF-β1 ELISA was performed as follows: primary pericytes were seeded at 150,000 cells per well in 6-well tissue-culture plates and cultured in 0.75% fetal bovine serum–supplemented DME 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 h after seeding. A mammalian protein extraction reagent (M-PER) cell lysis buffer supplement with protease inhibitor (Complete Mini) was used for lysate preparation (Roche). ELISA was performed according to kit instructions. Active TGF-β1 concentrations were calculated by interpolating values onto a standard curve generated with TGF-β1 accompanying the kit. For qPCR expression analyses, fold change was calculated using the ΔΔ cycle threshold method, in which WT at 0 pg/ml was the reference sample, and *GAPDH* was the reference gene. Sample preparation for qPCR expression analyses were as follows: serum-starved primary pericytes were seeded at 100,000 cells per well in fibronectin-coated 6-well tissue-culture plates in triplicate. Active TGF-β1 purchased from PeproTech was added to serum-starved pericytes at final concentrations of 0, 50, and 5,000 pg/ml. Cells were then incubated for 17 h at 37°C. RNA was harvested using TRIZOL reagent (Sigma-Aldrich). cDNA was synthesized using iScript (Bio-Rad Laboratories). 12.5 ng cDNA was used per 96-well PCR plate well, with each tissue-culture plate well represented in three individual PCR plate wells. The following primer sets were used for qPCR or RT-PCR: *CTGF* forward, 5′-AGCCTCAAACTCCAAACACC-3′, and reverse, 5′-CAACAGGGATTT-GACCAC-3′; *PAI-1* forward, 5′-GACACCCTCAGCATGTTCATC-3′, and reverse, 5′-AGGGTTGCACTAAACATGTCAG-3′; *BIGH3* forward, 5′-TGAT-AAGAGGGGACGGTTTG-3′, and reverse, 5′-ATTGGTGGGAGCAAAAA-CAG-3′; and *GAPDH* forward, 5′-AGAAGGCTGGGGCTCATTTG-3′, and reverse, 5′-AGGTCGGAGTCAACGGATTTG-3′.

To assess the effect of SPARC on SPARC$^{-/-}$ pericyte transcription, pericytes were cultured for 72 h in the presence or absence of recombinant SPARC or BSA control. Media were replaced with fresh SPARC- or BSA-containing media every 24 h before RNA extraction.

RT-PCR

Cells were incubated in 0.75% fetal bovine serum–supplemented DME overnight before RNA extraction and cDNA synthesis. The following primer sets were used for RT-PCR: *endoglin* (L-endoglin) forward, 5′-GCACTCTG-GTACATCTATTCTCACACACGTGG-3′, and reverse, 5′-GGGCACTACG-CCATGCTGCTGGTGG-3′; *SPARC* forward, 5′-CTGCGTGTGAAGAAG-ATCCA-3′, and reverse, 3′-TGGGACAGGTACCCATCAAT-3′; ALK5 forward, 5′-GGCGACGGCATTACAGTGTT-3′, and reverse 5′-TGTACATAC-AAATGGCCTGT-3′; *TβRII* forward, 5′-GCAAGTTTTGCGATGTGAGA-3′, and reverse, 5′-GGTATCTTCCAGAGTTGAAGC-3′; *TGF-β1* forward, 5′-TTG-CTTCAGCTCCCACAGAGA-3′, and reverse, 5′-TGGTTGTAGAGGGCAA-GGAC-3′; αV integrin: *itgav* forward 5′-GGGTGATCATCTTGGCAGTT-3′, and reverse, 5′-GAACTTGGAGCGGACAGAAG-3′; β1 integrin: *itgb1* forward, 5′-GTGACCCATTGCAAGGAGAAGGAC-3′, and reverse 5′-GTC-ATGAATTATCATTAAAAGTTT-3′; β3 integrin: *itgb3* forward, 5′-CTGGTGTT-TACCGATGCCAAG-3′, and reverse, 5′-TGTTGAGGCAGGTGGCATTGA-AGG-3′; β6 integrin: *itgb6* forward, 5′-CCGGCTGGCCAAAGAGATGT-3′, and reverse, 5′-AGTTAATGGCAAAATGTGCT-3′; RPS6: *rps6* forward, 5′-AA-GCTCCGCACCTTCTATGAGA-3′, and reverse, 5′-TGACTGGACTCAGA-CTTAGAAGTAGAAGC-3′; and β-actin: *actb* forward, 5′-ATATCGCTGCGCT-GGTCGTC-3′, and reverse, 5′-AGGATGGCGTGAGGGAGAGC-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 DME for 17 h before being lysed in 300 µl sample buffer (62.5 mM Tris-HCl, pH 6.8, at 25°C, 2% SDS, 10% glycerol, 50 mM DTT, and 0.01% bromophenol blue). Lysates were subjected to SDS-PAGE and Western blotting for tSMAD2 and pSMAD2 (serine 465/467) immediately thereafter.

Immunoprecipitation

10T1/2 cells were lysed in modified radioimmunoprecipitation assay buffer (0.5% deoxycholate, 0.5% SDS, 1% Triton X-100, 10 mM sodium phosphate, pH 7.2, 150 mM sodium chloride, and protease inhibitor [Complete Mini]). Pericytes were lysed in a milder buffer containing 1% NP-40, 10 mM sodium phosphate, pH 7.2, 150 mM sodium chloride, and protease inhibitor (Complete Mini). Lysis was performed on serum-starved adherent cells after washing with chilled PBS. Cells were scraped using 1 ml modified radioimmunoprecipitation buffer. Lysates were allowed to rotate at 4°C on a nutator for 1 h and then vortexed several times before centrifuging at 13,000 rpm for 10 min to pellet any insoluble material. Lysates were then precleared with protein A/G beads (Thermo Fisher Scientific). 200 µg cellular protein in 1 ml lysis buffer was used per immunoprecipitation reaction. 1 µg of the appropriate IgG was added with 20 µl protein A/G bead slurry to each sample; each sample was then allowed to rotate overnight at 4°C on a nutator. Immunoprecipitated complexes were washed twice in lysis buffer and 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 Systems), or serum

(EastCoast Bio), blocked, and incubated with recombinant endoglin or recombinant SPARC. Bound endoglin or SPARC was detected with anti-endoglin (MJ7/18) or anti-SPARC (mAb 303) antibodies or detected with horseradish peroxidase–conjugated secondary IgG. Assays were developed using tetramethylbenzidine reagent (Thermo Fisher Scientific). Samples were added in triplicate, and the experiment was repeated three times.

Surface protein labeling

Primary pericytes were grown to 80% confluency and then switched to 0.75% fetal bovine serum–supplemented DME. Cells were then labeled with a cell surface protein isolation kit (Sulfo-NHS-SS-Biotin; Thermo Fisher Scientific) according to the manufacturer's instructions. 4–10-cm dishes per pericyte genotype were used per fractionation. Fractionations were performed twice with identical results.

Image acquisition

Epifluorescent images were taken using a microscope (Eclipse E600; Nikon) and a camera (CoolSNAP HQ; Photometrics). Images were acquired and analyzed using NIS-Elements software. For visualization of immunofluorescently stained cells, images were thresholded so as to not include a signal caused by the nonspecific binding of the fluorophore-conjugated secondary antibody alone and analyzed as JPEG 2000 files. Confocal images were taken using either a TCS-SP5 confocal microscope (Leica) or an Eclipse TE2000E confocal microscope (Nikon). Leica images were taken using the Imaging Application for Confocal SP5 software (Leica). Images were saved as Leica Image Files (.LIF) and analyzed using ImageJ software (National Institutes of Health). Contrast and brightness were adjusted equally in all channels using Photoshop (CS3 Extended; Adobe). Nikon images were taken using a camera (CoolSNAP ES) and EZ-C1 3.8 software (Photometrics). Images were saved in the native ICS/IDS format. Images were processed using NIS-Elements software. Channels were thresholded so as to not include autofluorescence from the assay medium (10% FBS in DME containing phenol red). Nikon epifluorescent and confocal objectives (plan fluorite) had the following numerical apertures: 10×, 0.3; 20×, 0.5; 40×, 0.75; and 100×, 1.3 in oil. Leica confocal images were taken using a 63× objective with a 1.4 numerical aperture in oil. Fluorescent staining was performed using cyanine (Cy3) or fluorescein isothiocyanate–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.).

Statistics

Student's *t* test analysis or analysis of variance was performed for all experiments.

Online supplemental material

Fig. S1 shows vinculin and phalloidin staining used to assess focal adhesion formation in primary pericytes. Fig. S2 shows exogenous SPARC blocked the anti-TGF-β–induced enhancement of *SPARC*$^{-/-}$ pericyte migration. Fig. S3 shows that *SPARC*$^{-/-}$ pericytes exhibit enhanced basal TGF-β activity. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201011143/DC1.

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