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Role of secreted modular calcium-binding protein 1 (SMOC1) in transforming growth factor β signalling and angiogenesis

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Aims Secreted modular calcium-binding protein 1 (SMOC1) is a matricellular protein that potentially interferes with growth factor receptor signalling. The aim of this study was to determine how its expression is regulated in endothelial cells and its role in the regulation of endothelial cell function.

Methods and results SMOC1 was expressed by native murine endothelial cells as well as by cultured human, porcine, and murine endothelial cells. SMOC1 expression in cultured cells was increased by hypoxia via the down-regulation of miR-223, and SMOC1 expression was increased in lungs from miR-223-deficient mice. Silencing SMOC1 (small interfering RNA) attenuated endothelial cell proliferation, migration, and sprouting in *in vitro* angiogenesis assays. Similarly endothelial cell sprouting from aortic rings *ex vivo* as well as postnatal retinal angiogenesis *in vivo* was attenuated in SMOC1$^{+/-}$ mice. In endothelial cells, transforming growth factor (TGF)-β signalling via activin-like kinase (ALK) 5 leads to quiescence, whereas TGF-β signalling via ALK1 results in endothelial cell activation. SMOC1 acted as a negative regulator of ALK5/SMAD2 signalling, resulting in altered α2 integrin levels. Mechanistically, SMOC1 associated (immunohistochemistry, proximity ligation assay, and co-immunoprecipitation) with endoglin; an endothelium-specific type III auxiliary receptor for the TGF-β super family and the effects of SMOC1 down-regulation on SMAD2 phosphorylation were abolished by the down-regulation of endoglin.

Conclusion These results indicate that SMOC1 is an ALK5 antagonist produced by endothelial cells that tips TGF-β signalling towards ALK1 activation, thus promoting endothelial cell proliferation and angiogenesis.

Keywords Angiogenesis • Endoglin • Extracellular matrix • Matricellular protein • Transforming growth factor beta

1. Introduction

Vascular cells secrete several matricellular proteins that regulate cell-matrix interactions, cell adhesion, spreading, proliferation, invasion, angiogenesis, as well as epithelial-to-mesenchymal transition.[1] Deciphering the function of each matricellular protein is complicated by the fact that their roles vary depending on the context and the dynamic tissue conditions that accompany homeostasis and repair.[2] Secreted modular calcium-binding protein 1 (SMOC1) belongs to a family of matricellular proteins that also include secreted protein acidic and rich in cysteine (SPARC; also known as BM40 or osteonectin), as well as SMOC2 and testican-1.[3] Currently, SPARC is the best characterized member of this class and has been shown to interfere with the receptor-mediated signalling of several growth factors including platelet-derived growth factor,[4] vascular endothelial cell growth factor (VEGF),[5] fibroblast growth factor 2,[6] insulin-like growth factor 1,[7] and transforming growth factor (TGF)-β.[8]

SMOC1 is highly expressed during development[9,10] and in adult animals is generally expressed in the basement membrane of different tissues.[9,11–13] There, SMOC1 can interact with laminins,[11] CRP, fibulin-1, vitronectin,[14] transglutaminase 2,[15] and tenascin-C.[11,16] Little is known about the role of SMOC1 in physiology or pathophysiology, but its expression is increased in some forms of cancer,[16,17] and

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mutations within the SMOC1 gene cause the Waardenburg anophthalmia syndrome.[10,18] Interestingly, a whole genome scan for loci contributing to arterial stiffness in African Americans identified SMOC1 as being potentially linked to altered vascular structure.[19] The homologous protein, SMOC2, has been reported to increase endothelial cell proliferation and tube formation by a mechanism potentially related to growth factor responsiveness.[20,21] SMOC1 may well interfere with growth factor signalling in a similar manner as the *Xenopus* as well as the *Drosophila* orthologues of SMOC1 were found to antagonize TGF-β and BMP signalling.[22,23] Also, in mesangial cells, SMOC1 was characterized as a nitric oxide (NO)-regulated gene that affects TGF-β signalling.[24] Given the importance of TGF-β signalling in vascular homeostasis, the aim of this investigation was to assess the role played by SMOC1 in the regulation of endothelial cell function and the consequences of its knockdown on vascular signalling and angiogenesis.

2. Methods

2.1 Animals

Wild-type (C57BL/6) mice were from Charles River (Sulzfeld, Germany) and B6D2-Smoc1<Tn(sb-lacZ,GFP)PV384Jtak>/JtakRbrc mice (SMOC1$^{+/-}$) from the RIKEN BioResource Center (Tsukuba, Japan). miR-223 knockout mice (miR-223$^{y/-}$)[25] were provided by Fernando Camargo (Boston, USA). Mice (8 weeks of age) were kept either under normoxic (21% O_2) or hypoxic (10% O_2) conditions in ventilated chambers for 21 days. All animals were housed in conditions that conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23). Both the University Animal Care Committee and the Federal Authority for Animal Research at the Regierungspräsidium Darmstadt (Hessen, Germany) approved the study protocol (#F28/21and #F28/36). Age-, gender-, and strain-matched animals (usually littermates) were used throughout. For the isolation of organs, mice were sacrificed using 4% isoflurane in air and subsequent exsanguination.

2.2 Cell culture

Porcine aortae were obtained from a local abattoir and umbilical veins from a local hospital. Human umbilical vein and porcine aortic endothelial cells were isolated and cultured as described,[26,27] and used up to Passage 3. The use of human material in this study conforms to the principles outlined in the Declaration of Helsinki, and the isolation of endothelial cells was approved in written form by the ethic committee of the Goethe University.

2.3 Statistics

Values are expressed as the mean ± SEM, in some cases data were normalized with respect to control which was set to 1 or 100% and where possible error bars were included to indicate experimental variability between control experiments. Statistical evaluation was performed using Student's *t*-test (two-tailed) for analysis between two groups containing normally distributed data and one- or two- way ANOVA followed by the Bonferroni *t* test for comparisons between multiple groups (as indicated), and values of $P < 0.05$ were considered statistically significant.

For detailed methods, see Supplementary material online.

3. Results

3.1 SMOC1 is secreted by endothelial cells

SMOC1 was detected in the endothelium-intact murine aorta and was restricted to the CD31-positive endothelial cell layer and absent from the vascular smooth muscle cell layers (*Figure 1A*). Consistent with its classification as a matricellular protein, low levels of SMOC1 were detected in cell lysates (*Figure 1B*). The majority of the SMOC1 generated by primary cultures of human umbilical vein and porcine aortic endothelial cells was secreted and detectable in the cell supernatant (*Figure 1B*) as well as on the surface of non-permeabilized cells (*Figure 1C*). The siRNA-mediated down-regulation of SMOC1 in human endothelial cells effectively decreased surface levels of the protein as well as its presence in the cell supernatant (*Figure 1D*).

3.2 Regulation of endothelial SMOC1 expression

Little is known about the factors regulating SMOC1 levels, but the expression of the protein in mesangial cells can be modulated by NO.[24] In a confluent layer of human endothelial cells, the NO donor, diethylenetriamine NONOate, also attenuated SMOC1 expression while IL-1β, tumour necrosis factor-α, as well as TGF-β1 significantly increased SMOC1 levels (*Figure 2A*). Given the reported role of SMOC2 in growth factor signalling, which can be affected by the O_2 partial pressure, the consequences of hypoxia on SMOC1 expression were assessed. Hypoxia (1% O_2) increased SMOC1 mRNA levels in cultured human endothelial cells (*Figure 2B*), as well as the secretion of the protein into the cell supernatant (*Figure 2C*). This phenomenon was also observed *in vivo* as SMOC1 expression was also increased in lungs from mice exposed to hypoxia (10% O_2) for 3 weeks (*Figure 2D*).

The human SMOC1 promoter contains three putative HIF1α binding sites, two of which are in close proximity to the transcriptional start site (−520 and −268 bp). However, the overexpression of constitutively active HIF1α or 2α mutants in HEK-293 cells failed to increase SMOC1 protein levels (see Supplementary material online, *Figure S1A*). *In silico* analysis of the SMOC1 3′ untranslated region (UTR; Targetscan, miRDB, and miRanda) revealed a potential seeding sequence (6 bp of complementarity) for miR-223. Co-transfecting HEK-293 cells with increasing concentrations of precursor (pre)-miR-223 and a luciferase construct containing the putative miR-223 binding sequence within the 3′UTR of SMOC1, resulted in a concentration-dependent decrease in luciferase activity (*Figure 2E*). No effect of pre-miR-223 was detected when the binding sequence was mutated (see Supplementary material online, *Figure S1B*), indicating that SMOC1 is directly targeted by miR-223.

Although miR-223 is expressed in native endothelial cells, levels rapidly decrease once the cells are cultured.[28] The microRNA could however still be detected in primary cultures of endothelial cells and miR-223 levels were significantly decreased following exposure to hypoxia (*Figure 2F*). Fitting with the regulation of SMOC1 by a miR-223-dependent mechanism the overexpression of pre-miR-223 abolished SMOC1 expression under basal conditions as well as in cells exposed to hypoxia (*Figure 2G*). Importantly, the application of hypoxia to third-passaged human endothelial cells, which expressed no detectable miR-223, failed to increase SMOC1 expression (see Supplementary material online, *Figure S1C*).

The miR-223 locus is located on the X chromosome[25] and to confirm our results in native endothelial cells SMOC1 expression was assessed in lungs from male wild-type (miR-223$^{y/+}$) and miR-223 knockout (miR-223$^{y/-}$) littermates. SMOC1 expression was higher in lungs from miR-223$^{y/-}$ mice than wild-type littermates kept under normoxic conditions but unlike the wild-type mice, exposure to hypoxia failed to further increase protein expression (*Figure 2H*).

3.3 Link between SMOC1 and angiogenesis

Given that miR-223 has been classified as an anti-angiogenic microRNA,[28] we hypothesized that its target, SMOC1, should promote

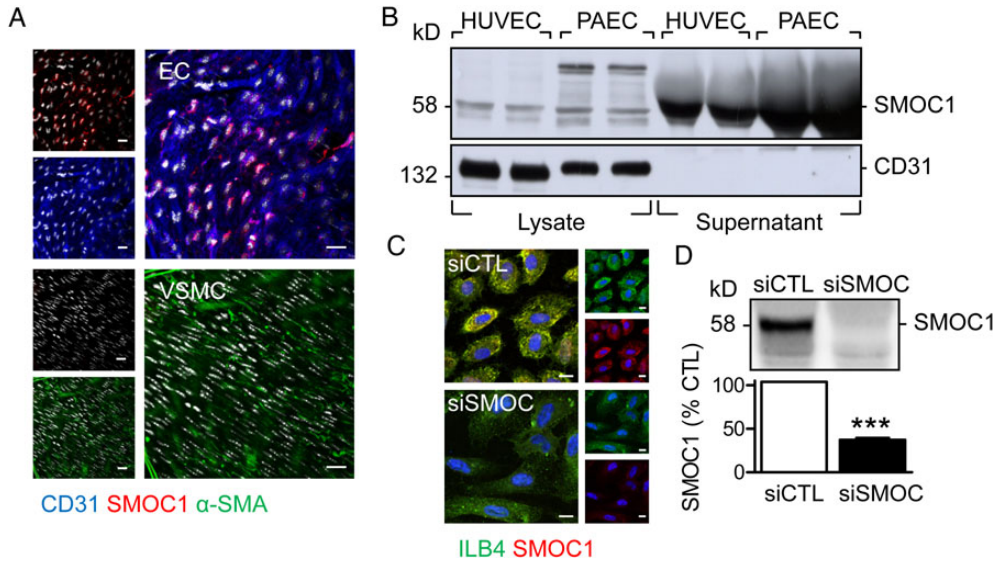

Figure 1 SMOC1 is secreted by native and cultured endothelial cells. (*A*) Co-localization of SMOC1 (red) with CD31 (green) in an *en face* mouse aorta preparation. Smooth muscle cells were stained for smooth muscle actin (α-SMC; green) and nuclei are white; bar = 20 μm. Similar results were obtained using three additional aortae. (*B*) SMOC1 expression in cell lysates and culture media (supernatant) from cultured human umbilical vein endothelial cells (HUVEC) and porcine aortic endothelial cells (PAEC). Similar results were obtained using three additional cell batches. (*C*) SMOC1 surface expression in non-permeabilized endothelial cells treated with a control siRNA (siCTL) or siRNA directed against SMOC1 (siSMOC); SMOC1 = red, Isolectin B4 (IB4) = green; bar = 10 μm. Similar results were obtained using four additional cell batches. (*D*) SMOC1 levels in the supernatants of human endothelial cells treated with a control siRNA (siCTL) or siRNA directed against SMOC1 (siSMOC). The graph represents data from 10 independent experiments each using a different batch of endothelial cells; ***$P < 0.001$ vs. siCTL (*t*-test).

angiogenesis. To determine whether or not this was the case, the consequences of SMOC1 down-regulation were studied in cultured endothelial cells. The siRNA-mediated down-regulation of SMOC1 attenuated the migration of endothelial cells on laminin and collagen (*Figure 3*B) as was endothelial cell proliferation in the presence of 5% serum (*Figure 3C*). Endothelial cell tube formation (*Figure 3D*) was also attenuated in SMOC1-deficient cells seeded onto Matrigel.

SMOC1$^{-/-}$ mice usually die shortly after birth; therefore, endothelial cell sprouting was studied in SMOC$^{+/+}$ and SMOC1$^{+/-}$ littermates. As in the *in vitro* studies, the sprouting of endothelial cells from SMOC$^{+/-}$ aortic rings was significantly attenuated (*Figure 4A*). Similarly, the postnatal development of the retinal vasculature (Isolectin B4 positive cells) was significantly delayed in SMOC1$^{+/-}$ mice (*Figure 4B*). Because of the lack of blood flow in the developing retina, the endothelial cells at the angiogenic front are subjected to hypoxia. Consistent with the *in vitro* findings on the regulation of SMOC1 by hypoxia, its expression was highest in endothelial cells at the angiogenic front (*Figure 4C*).

3.4 Role of SMOC1 in regulating TGF-β signalling in endothelial cells

Next we focused on determining the consequences of SMOC1 down-regulation on TGF-β signalling as previous studies[22,24] have suggested a link between the two.

In response to agonist stimulation, the TGF-β receptor (TβR) type II heterodimerizes in endothelial cells with activin receptor-like kinase (ALK) 1 or ALK5 resulting in the phosphorylation of SMAD1/5 or SMAD2, respectively. In quiescent endothelial cells, TGF-β signal transduction is predominantly mediated by ALK5 and phosphorylation of SMAD2 to regulate the expression of target genes such as plasminogen activator inhibitor (PAI)-1.[29,30] In mink lung epithelial cells that constitutively expressed a PAI1-promoter luciferase construct, TGF-β1 significantly increased reporter gene activity (*Figure 5A*). SMOC1 overexpression markedly decreased luciferase activity under basal conditions and attenuated the effect of exogenous TGF-β. The down-regulation of SMOC1 in endothelial cells elicited the opposite effect, i.e. increased the expression of PAI1 (*Figure 5B*). In the same cells, the expression of inhibitor of DNA binding 1 (ID1), which is regulated via ALK1, was significantly attenuated.

In endothelial cells maintained under control conditions, a basal phosphorylation of SMAD1/5 was detected and was attenuated by the down-regulation of SMOC1 (*Figure 5C*). The stimulation of cells with TGF-β had little effect on SMAD1/5 phosphorylation which remained attenuated in cells lacking SMOC1. The phosphorylation of SMAD2 was, on the other hand, increased by SMOC1 down-regulation to levels similar to those seen in TGF-β-stimulated cells (*Figure 5C*). The increase in SMAD2 phosphorylation (*Figure 5D*) as well as the altered expression of PAI1 and ID1 (*Figure 5E*) observed in SMOC1-deficient cells were normalized following the addition of exogenous SMOC1 in the form of conditioned medium from SMOC1-overexpressing HEK-293 cells. There was no effect of conditioned medium from GFP-expressing cells. These results indicate that the down-regulation of SMOC1 leads to an imbalance in endothelial cell TGF-β signalling that tends towards ALK5 activation.

TGF-β can regulate the expression of α2 integrin[31] and SMOC1 down-regulation was paralleled by a decrease in the expression of α2 integrin mRNA (*Figure 6A*). The latter phenomenon was rescued by the exogenous application of SMOC1 conditioned medium. The loss of SMOC1 also resulted in the attenuated surface expression of α2 integrin protein (*Figure 6B*), without affecting surface levels of β1, α5 or

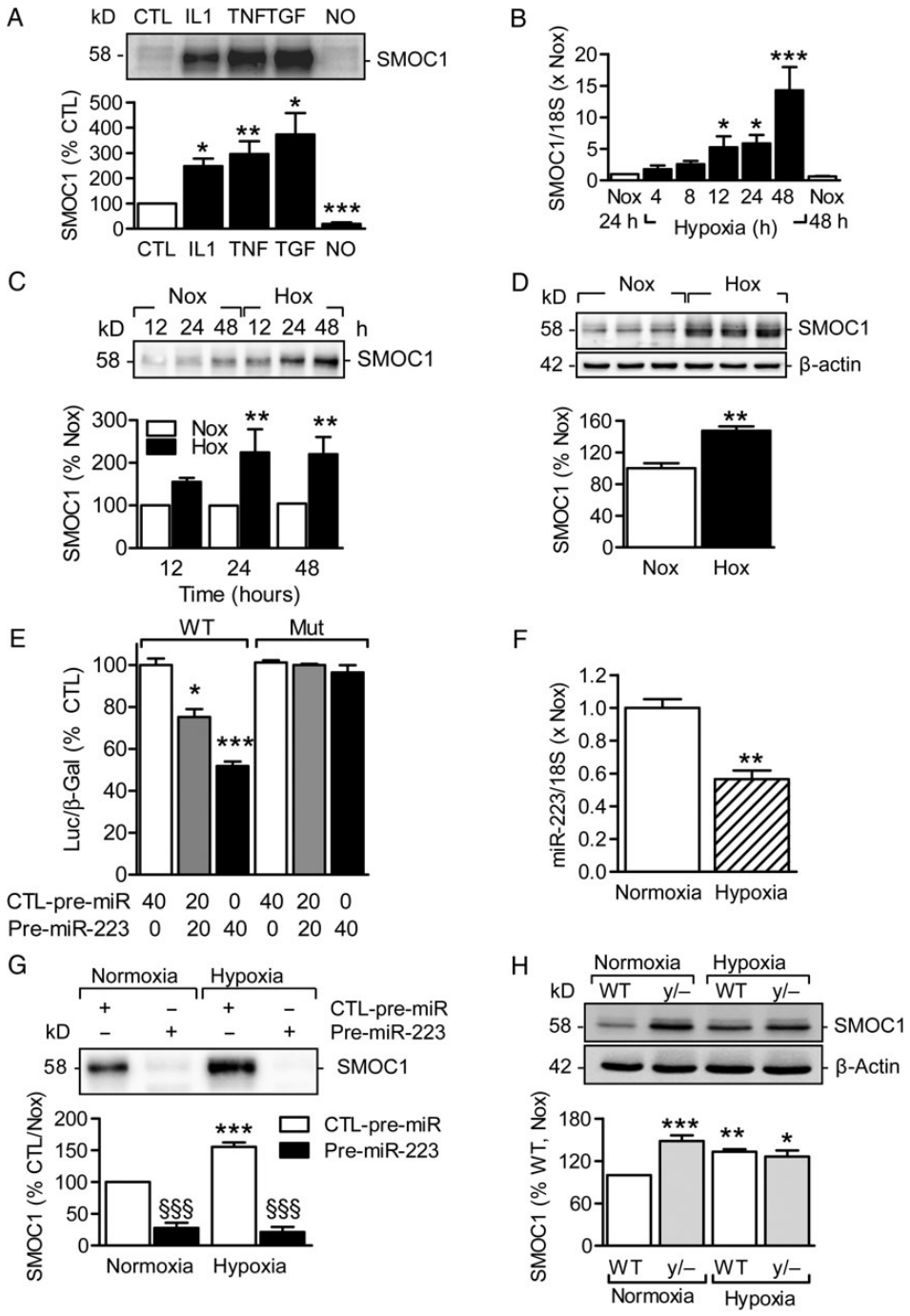

Figure 2 Regulation of SMOC1 expression. (*A*) SMOC1 expression in the supernatant from primary cultures of human endothelial cells treated with solvent (CTL), IL-1β (IL1, 1 ng/mL), TNF-α (TNF, 1 ng/mL), TGF-β1 (TGF, 10 ng/mL), or NONOate (NO, 200 μmol/L) for 8 h. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control (one-way ANOVA). (*B*) SMOC1 mRNA expression in human endothelial cells cultured under normoxic (Nox, 20% O_2) or hypoxic (Hox, 1% O_2) conditions for up to 48 h; $^{*}P < 0.05$, $^{***}P < 0.001$ vs. normoxia (one-way ANOVA). (*C*) SMOC1 protein secreted by human endothelial cells cultured under normoxic (Nox, 20% O_2) or hypoxic (Hox, 1% O_2) conditions for up to 48 h; $^{**}P < 0.01$ vs. time-controlled normoxia (two-way ANOVA). (*D*) SMOC1 expression in lungs from wild-type mice maintained in normoxia (Nox, 20% O_2) or hypoxia (Hox, 10% O_2) for 3 weeks; $^{**}P < 0.01$ normoxia (*t*-test). (*E*) Effect of overexpression of a control pre-microRNA (CTL-pre-miR) and pre-miR-223 on the activity of the wild-type (WT) or mutated SMOC1 3′UTR (Mut); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. CTL-pre-miR (two-way ANOVA). (*F*) Effect of hypoxia (1% O_2, 48 h) on the expression of miR-223 in primary cultures of human endothelial cells; $^{**}P < 0.01$ vs. CTL/normoxia (*t*-test). (*G*) Consequence of miR-223 overexpression on SMOC1 levels in the supernatants from human endothelial cells (Passage 3) maintained under normoxic (20% O_2) or hypoxic conditions (1% O_2) for 48 h; $^{***}P < 0.001$ vs. CTL/normoxia; $^{§§§}P < 0.001$ vs. CTL-pre-miR (two-way ANOVA). (*H*) SMOC1 protein expression in lungs from wild-type or miR-223$^{y/-}$ mice maintained under normoxic (20% O_2) or hypoxic (10% O_2) conditions for 3 weeks; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. wild-type/normoxia (two-way ANOVA). The graphs summarize data from 4 to 11 independent experiments each using a different batch of human endothelial cells (*A*–*C* and *E*–*G*) or six mice per group (*D* and *H*).

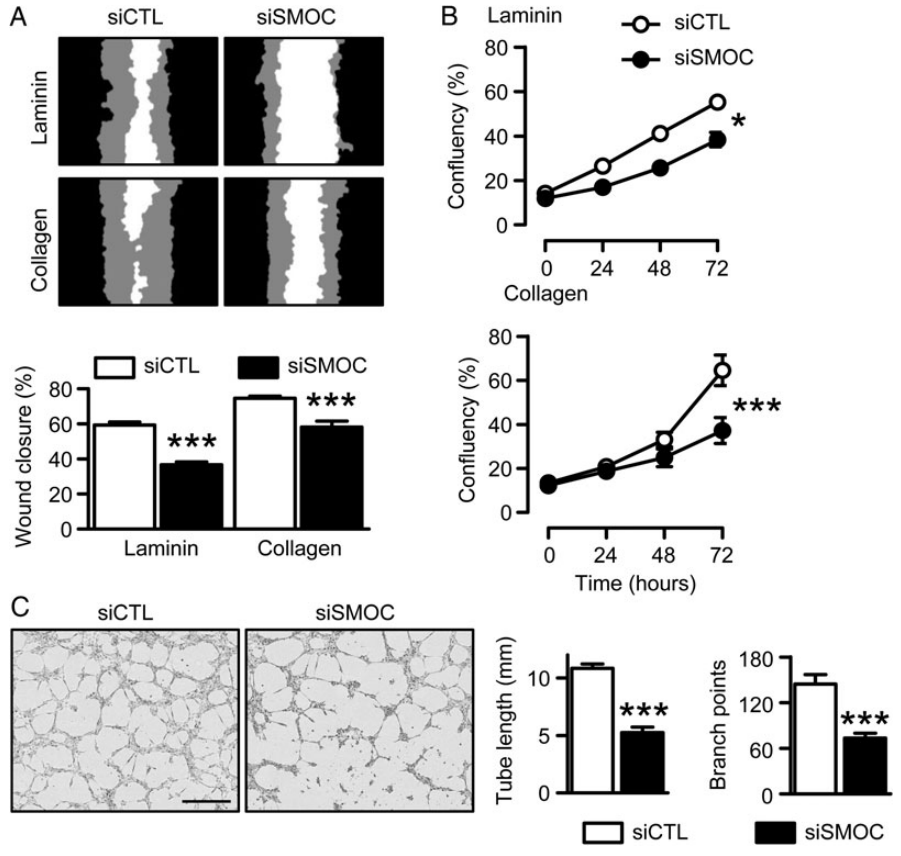

Figure 3 Consequences of SMOC1 knockdown on endothelial cell function. Primary human endothelial cells were treated with a control siRNA (siCTL) or siRNA directed against SMOC1. (*A*) Migration in a scratch wound assay. ***$P < 0.001$ vs. siCTL (one-way ANOVA). (*B*) Proliferation in the presence of 5% FCS. *$P < 0.05$, ***$P < 0.001$ vs. siCTL (two-way ANOVA repeated measures). (*C*) Consequence of SMOC1 knockdown on tube formation; bar = 400 μm; ***$P < 0.001$ vs. siCTL (*t*-test). The graphs summarize data from 5 to 10 different cell batches.

α6 integrins (Data not shown). The pharmacological inhibition of ALK5, using SD-208, prevented the decrease in α2 integrin (*Figure 6B*) and normalized the defective migration (*Figure 6D*) and tube formation (*Figure 6E*) that were associated with the down-regulation of SMOC1. The inhibition of ALK1 with LDN193189 failed to prevent the SMOC1 siRNA-induced decrease in α2 integrin expression, or tube formation. The compound however attenuated endothelial cell migration under control conditions. There are of course additional ALK1 and ALK5 targets in endothelial cells and the down-regulation of SMOC1 decreased the expression of ET-1[32] and IL-8,[33] both of which are ALK5 targets, at the same time as increasing the expression of the anti-apoptotic gene and ALK1 target BCL2[34] (see Supplementary material online, *Figure S2*). In all cases, rescue experiments in which SMOC1 was reintroduced to SMOC1-deficient cells normalized gene expression.

TGF-β1 amplifies its signalling by inducing its own expression[35] and SMOC1 down-regulation was previously found to decrease the expression of TGF-β1.[24] These findings could be reproduced in SMOC1-deficient endothelial cells, which expressed lower levels of TGF-β1 mRNA (68 ± 12% of mRNA expressed in cells treated with control siRNA; $P < 0.05$, $n = 8$).

3.5 Association of SMOC1 with endoglin

The ability of SMOC1 to decrease TGF-β expression and act as a negative regulator of ALK5 signalling was reminiscent of the actions of endoglin. The latter protein is highly expressed in proliferating endothelial cells[36] and is required for the suppression of ALK5 signalling and the facilitation of ALK1 signalling.[37] Therefore, a possible interaction between SMOC1 and endoglin was addressed.

In retinas from 5-day-old wild-type mice, SMOC1 and endoglin were co-localized to endothelial cells at the angiogenic front (*Figure 7A*). Using an *in situ* proximity ligation assay, it was possible to demonstrate the physical association of SMOC1 with endoglin in endothelial cells (*Figure 7B*). Correspondingly, the proximity signal was attenuated by the siRNA-mediated down-regulation of endoglin (by ~60%; see Supplementary material online, *Figure S3A*). In addition, endoglin was coprecipitated with SMOC1 from human endothelial cells (*Figure 7C*). No interaction was detected in cells treated with siRNAs directed against either SMOC1, endoglin, or both proteins. Endoglin deletion also prevented the phosphorylation of SMAD2 (*Figure 7D*), as well as the decrease in α2 integrin expression (*Figure 7E*) and the impaired endothelial cell migration elicited by the loss of SMOC1 (*Figure 7F*). Decreasing the expression of endoglin in human endothelial cells did not affect the expression of SMOC1, ALK1, or ALK5, nor did the down-regulation of SMOC1 affect endoglin, ALK1, or ALK5 levels (see Supplementary material online, *Figure S3B*). These findings indicated that the actions of SMOC1 rely on the presence of endoglin. Given that SMOC1 is a secreted protein it most likely interacts with the extracellular domain of endoglin.

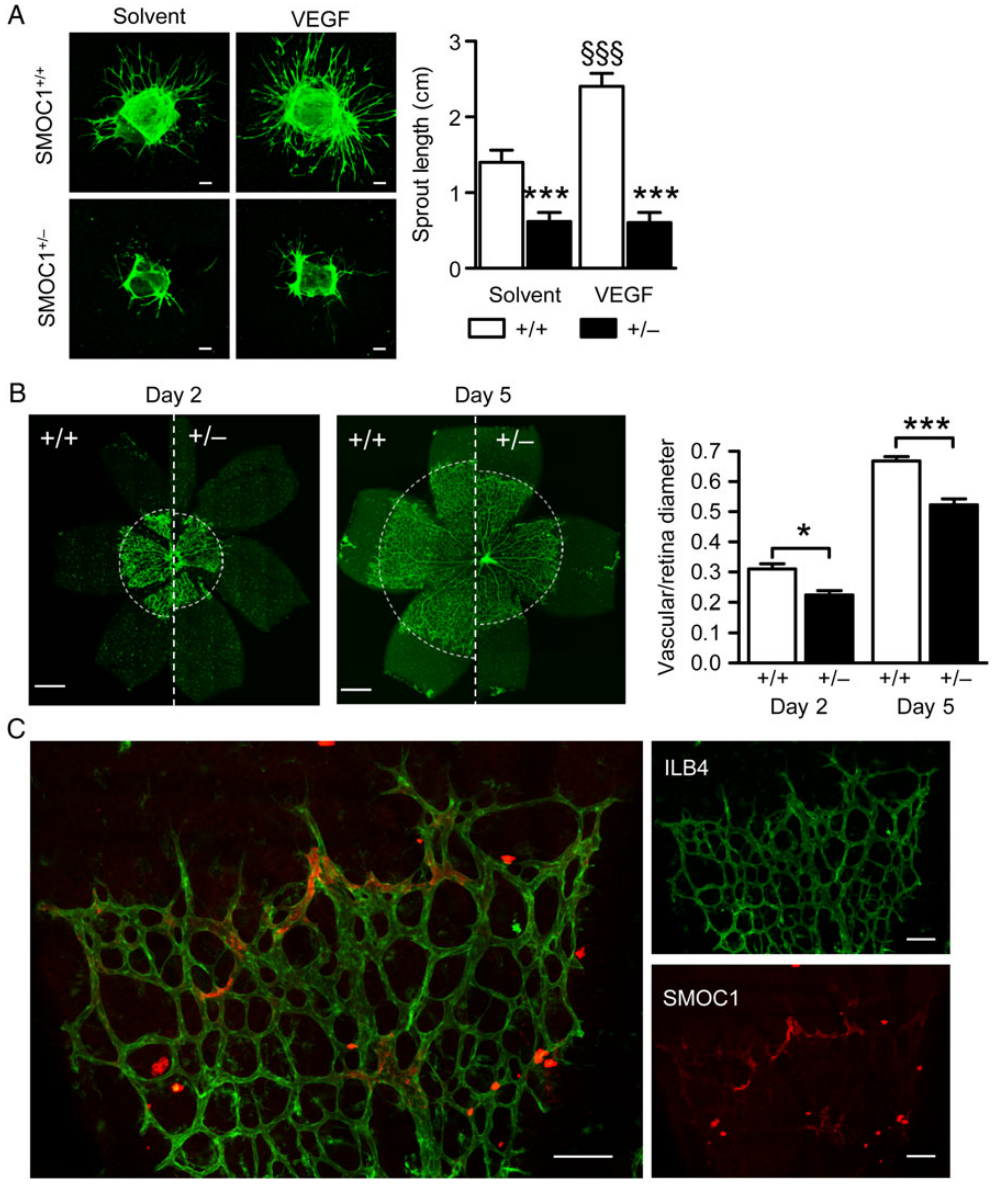

Figure 4 Consequences of SMOC1 down-regulation on angiogenesis. (*A*) Endothelial sprouting from aortic rings from SMOC$^{+/+}$ and SMOC$^{+/-}$ littermates, treated with solvent or VEGF (30 ng/mL); CD31 = green; bar = 200 μm. ***$P < 0.001$ vs. SMOC1$^{+/+}$ (two-way ANOVA); §§§$P < 0.001$ vs. sol (two-way ANOVA). (*B*) Comparison of retinal vascularization determined by Isolectin B4 staining (green) in SMOC$^{+/+}$ (+/+) and SMOC$^{+/-}$ (+/−) littermates 2 and 5 days after birth. The graphs summarize data from 4 to 11 different animals/group; *$P < 0.05$, ***$P < 0.001$ vs. SMOC$^{+/+}$ (two-way ANOVA). (*C*) SMOC1 (red) localization at the angiogenic front in the developing murine retina at postnatal day 5. The vasculature was visualized with Isolectin B4 (ILB4, green); bar = 100 μm. The image is representative of three additional retinas all from different animals.

4. Discussion

The results of the current study indicate that SMOC1 is secreted from vascular endothelial cells and that its expression can be increased by hypoxia via a mechanism linked to the down-regulation of miR-223. SMOC1 is highly expressed in proliferating endothelial cells and can regulate endothelial cell function at least partly by binding to endoglin and acting as a negative feedback regulator of ALK5 signalling. The result is that TGF-β signalling is tipped towards ALK1 activation, which leads to endothelial cell proliferation and angiogenesis.

Although there has been a lot of interest in the regulation of vascular homeostasis by matricellular proteins, and SMOC1 has been linked to altered arterial stiffness and structure,[19] little is known about the mechanisms regulating its expression or its potential role in the vasculature. We found that SMOC1 is generated and secreted by endothelial cells *in vivo* and *in vitro* and confirmed the regulation of SMOC1 expression by inflammatory cytokines and NO.[24] The finding that hypoxia can regulate the expression of SMOC1 is novel and fits well with an active role in the regulation of angiogenesis. The human SMOC1 promoter contains three putative HIF1α binding sites, two of which lie close to the transcriptional start site. However, the overexpression of constitutively active HIF1α or 2α mutants in HEK-293 cells failed to increase SMOC1 protein levels. Such findings indicated that the increase in SMOC1 observed under hypoxic conditions must occur independently

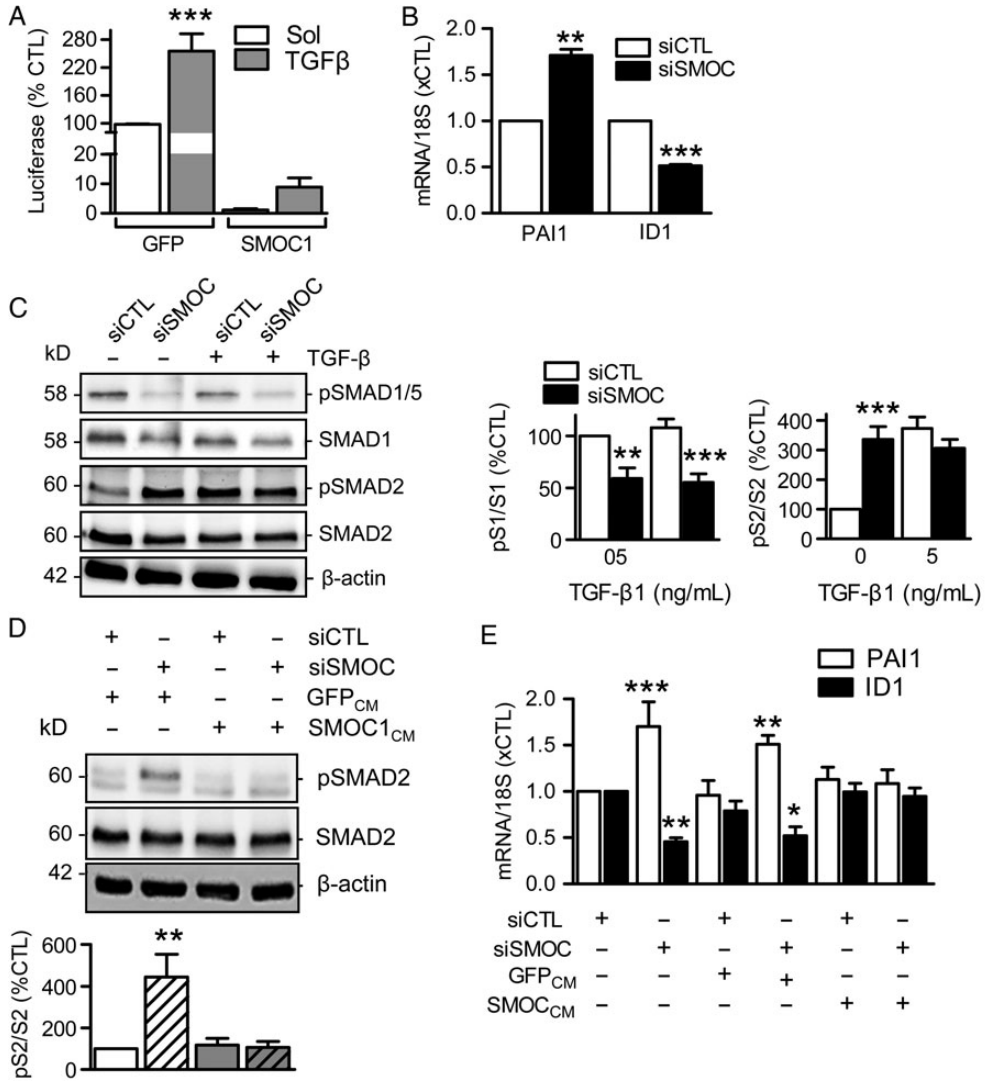

Figure 5 Effect of SMOC1 on TGF-β signalling. (*A*) Effect of the overexpression of green fluorescent protein (GFP) or SMOC1 on PAI1 promotor activity in cells treated with solvent (Sol) or TGF-β1 (10 ng/mL, 24 h, two-way ANOVA. (*B*) PAI1 and ID1 expression in primary human endothelial cells treated with a control siRNA (siCTL) or siRNA directed against SMOC1 (*t*-test). (*C*) SMAD1/5 and SMAD2 phosphorylation (ratio of phosphorylated to total protein) in primary endothelial cells treated with a control siRNA (siCTL) or siRNA directed against SMOC1 and incubated with solvent or TGF-β1 (5 ng/mL, 30 min, two-way ANOVA). (*D* and *E*) Comparison of the effects of conditioned medium from GFP overexpressing (GFP_{CM}) or SMOC1 overexpressing ($SMOC1_{CM}$) HEK-293 cells on endothelial cell SMAD2 phosphorylation (*D*, two-way ANOVA), as well as the expression of PAI1 and ID1 (*E*, two-way ANOVA). The graphs summarize data from 4 to 6 different cell batches; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. GFP/solvent (*A*), or vs. siCTL (*B*–*E*).

of HIF proteins. Given that several, so-called hypoxamiRs[38] are regulated by hypoxia and affect the cardiovascular system,[39] we screened the 3′UTR of SMOC and detected a potential seeding sequence for miR-223. Unlike the prototypical hypoxamiR, miR-210,[38] miR-223 levels decreased in response to hypoxia. The latter effect can most likely be attributed to an interaction between CCAAT/enhancer binding protein-α (C/EBPα) and HIF1α. Indeed, the miR-223 core promoter region contains C/EBPα response elements that positively regulate miR-223 promoter activity.[40] The fact that C/EBPα activity is negatively regulated by the binding of HIF1α,[41] an interaction that alters the function of the former,[42] can account for the down-regulation of miR-223 in cells exposed to hypoxia. A decrease in miR-223 could account for the hypoxia-induced increase in SMOC1 expression as co-transfection with increasing concentrations of miR-223 significantly attenuated the expression of a reporter gene construct containing the putative miR-223 binding sequence within the 3′UTR of SMOC1 mRNA. Mutation of the miR-223 binding sequence resulted in the loss of this effect, indicating that SMOC1 is a direct target of miR-223. Moreover, SMOC1 levels were decreased in cultured endothelial cells that overexpressed pre-miR-223 but increased in lungs from miR-223$^{y/-}$ mice. Interestingly, retinal angiogenesis is thought to be driven by a hypoxia-induced VEGF gradient,[43] and fitting with its regulation by hypoxia, SMOC1 was expressed mainly at the angiogenic front in the developing retinal vasculature of 5-day-old mice.

Similar to its close homologue SPARC, SMOC1 down-regulation differentially affected the adhesion of cells seeded onto different matrices.

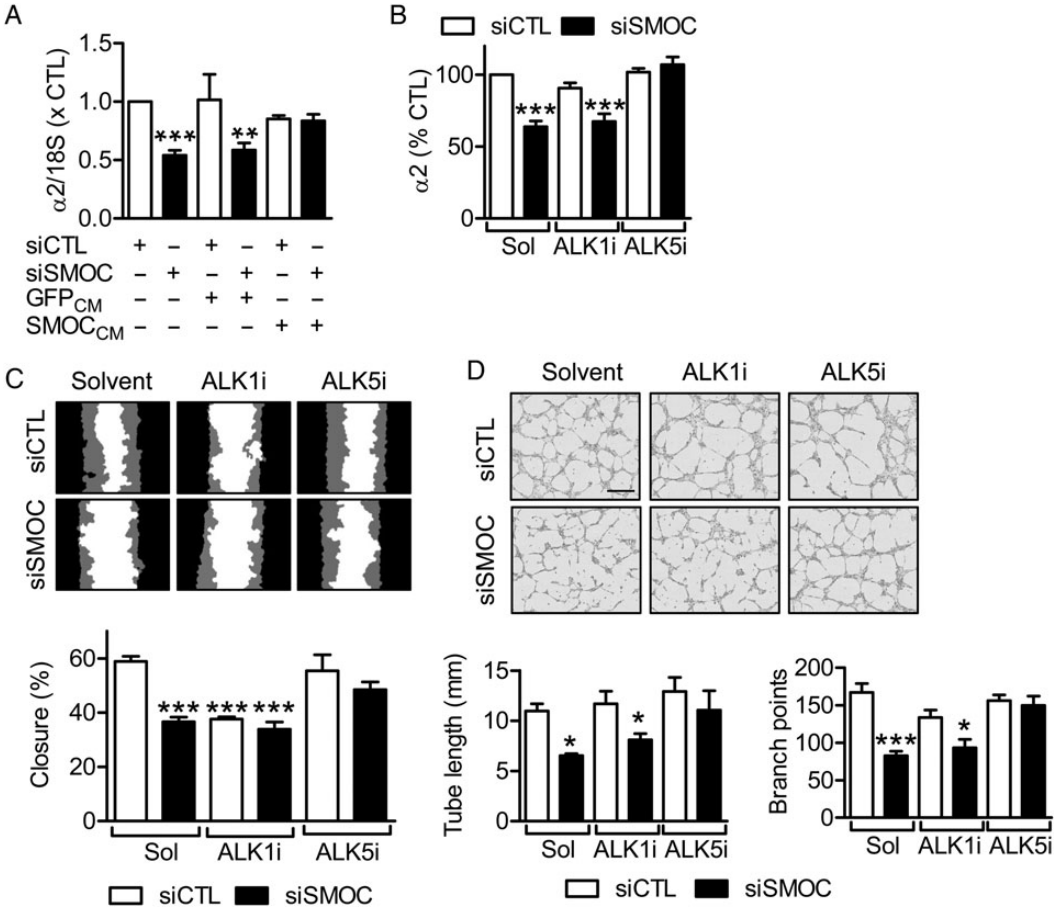

Figure 6 Effect of ALK1 and ALK5 inhibition on SMOC1-mediated endothelial cell function. Primary human endothelial cells were treated with a control siRNA (siCTL) or siRNA directed against SMOC1 48 h before being treated with either solvent (Sol), GFP conditioned medium (GFP$_{CM}$), SMOC1 conditioned medium (SMOC1$_{CM}$), LDN193189 (ALK1i; 100 nmol/L), or SD-208 (ALK5i; 100 nmol/L). (*A*) α2 Integrin mRNA expression. (*B*) The surface expression of α2 integrin as assessed by flow cytometry. (*C*) Cell migration on laminin over 24 h in a scratch wound assay. (*D*) Consequence of ALK1 or ALK5 inhibition on SMOC1-dependent tube formation; scale bar = 400 μm. The graphs summarize data from 4 to 6 different cell batches; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. siCTL/Sol (two-way ANOVA).

For example, while SMOC1 down-regulation attenuated endothelial cell adhesion on type I collagen, laminin-111 and Matrigel (Data not shown), there was no apparent effect on cells seeded onto fibronectin. This effect may be partly explained by integrin engagement as the effects of SMOC1 on adhesion were partially sensitive to EDTA. Of the integrins assessed (α2, β1, α5, and α6), the down-regulation of SMOC1 affected only the TGF-β target α2 integrin, suggesting that it acts as a modulator of integrin-matrix interactions at least partly by modulating integrin surface expression. However, integrin-mediated adhesion can also depend on the co-receptor function of heparan sulfate proteoglycans. Although the role of heparin binding was not addressed in the current study, the regulation of cell adhesion by SMOC1 in other cells has recently been attributed to its heparin binding activity.[44]

The effects of SMOC1 were not restricted to adhesion as endothelial cell proliferation, migration, and tube formation were all attenuated by SMOC1 down-regulation. These effects were not limited to cultured endothelial cells but could be reproduced using aortic rings from SMOC$^{+/-}$ mice and evident in delayed retinal angiogenesis in SMOC1$^{+/-}$ mice. Ours is not the first study to link SMOC1 with retinal defects as SMOC1 has been linked with Waardenburg anophthalmia which is associated with developmental eye defects.[45] miR-223 is also of interest with respect to angiogenesis as although it is known to be enriched in neutrophils, monocytes,[25] and platelets,[46,47] it is expressed in quiescent endothelial cells *in situ*—but rapidly down regulated in cultured endothelial cells.[28] These observations led to the proposal that miR-223 is a determinant of endothelial cell quiescence. In fact, angiogenesis was markedly accelerated in miR-223-deficient cells and miR-223$^{-/-}$ mice. At the molecular level, a decrease in miR-223 was found to be a prerequisite for β1 integrin expression and the initiation of growth factor receptor signalling.[28] The finding that SMOC1 is a miR-223 target and also a prerequisite for the sprouting of primary endothelial cells *in vitro*, as well as endothelial cell sprouting from aortic rings *ex vivo* and retinal angiogenesis *in vivo* strengthens the link between miR-223 and vascular homeostasis.

As a matricellular protein, SMOC1 may interfere with angiogenesis in a number of ways, i.e. by binding different extracellular matrix components or growth factors, or by altering their signalling. Given that SMOC1 has been linked to TGF-β signalling,[22,24] the consequences of SMOC1 overexpression and down-regulation on this pathway were investigated. In quiescent endothelial cells, TGF-β signal transduction

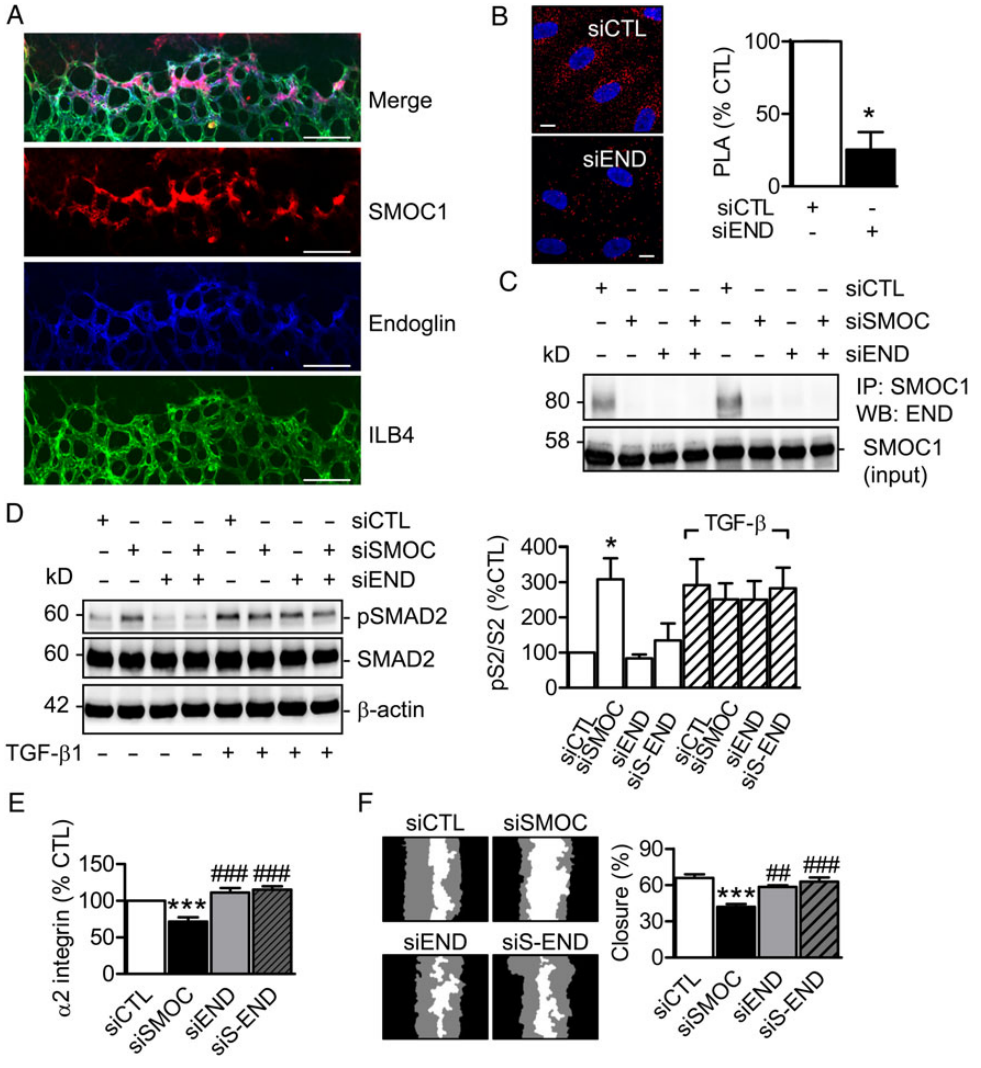

Figure 7 Link between SMOC1 and endoglin. (*A*) SMOC1 (red) and endoglin (blue) co-localization at the angiogenic front in the developing murine retina at postnatal day 5. The vasculature was visualized with Isolectin B4 (ILB4, green); bar = 100 μm. The image is representative of three additional retinas all from different animals. (*B*–*F*) Human endothelial cells were treated with a control siRNA (siCTL) or siRNAs directed against SMOC1 (siSMOC), endoglin (siEND), or a combination of both (siS-END). (*B*) Proximity ligation assay using SMOC1 and endoglin primary antibodies; bar = 20 μm. Similar results were obtained using three additional cell batches. The graph shows the normalized proximity ligation signal (PLA, fluorescent signal at 546 nm); $^{*}P < 0.05$ siCTL (*t*-test). (*C*) SMOC1 was immunoprecipitated (IP) from BS3-treated endothelial cells and the co-precipitation of endoglin (END) assessed by western blotting (WB). Similar results were obtained using three additional cell batches. (*D*) SMAD2 phosphorylation quantified as the level of phosphorylated vs. total SMAD2 protein (pS2/S2, two-way ANOVA). (*E*) Surface expression of α2 integrin assessed by flow cytometry (one-way ANOVA). (*F*) Endothelial cell migration on laminin 24 h after placing the scratch wound (one-way ANOVA). The graphs summarize data from four different cell batches; $^{*}P < 0.05$, $^{***}P < 0.001$ vs. siCTL, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. siSMOC.

is predominantly mediated by the type I TGF-β receptor, ALK5, and phosphorylation of SMADs 2 and 3 leading to the expression of target genes such as PAI1. Endothelial cell proliferation however is associated with the switch to predominantly ALK1/SMAD1 and SMAD5 signalling[29,37] and the expression of target genes like ID1. The siRNA-mediated down-regulation of SMOC1 in cultured endothelial cells was associated with the activation of ALK5 signalling—detected as an increase in SMAD2 phosphorylation that was similar to levels in TGF-β-stimulated cells. In the same cells, the ALK1-dependent phosphorylation of SMAD1 was attenuated. Moreover, a rescue experiment in which conditioned medium from SMOC1-overexpressing HEK-293 cells was added to SMOC1-deficient endothelial cells decreased SMAD2 phosphorylation to basal levels. Similar approaches were used to demonstrate that SMOC1 negatively regulates the expression of the ALK5/SMAD2 target; PAI1, but increases the expression of the ALK1/SMAD1/5 target, ID1. Thus, the presence of SMOC1 alters the predominant TGF-β signalling pathway in endothelial cells from ALK5 towards ALK1, i.e. from a non-angiogenic to a pro-angiogenic state. TGF-β can also regulate the expression of α2 integrin[31] and together with laminins maintain cell polarity in an integrin α2-dependent manner.[48] Our hypothesis that SMOC1 interferes with ALK5 would mean that the down-regulation of SMOC1 should alleviate the inhibition and increase ALK5 signalling. Indeed, the decrease in α2 integrin levels, as well as endothelial cell adhesion, migration, and tube formation elicited by SMOC1 down-regulation were all normalized by ALK5 inhibition.

How could SMOC1 interfere with ALK5 signalling? TGF-β1 amplifies its signalling by inducing its own expression,[35] and SMOC1 also decreased the expression of TGF-β1. However, even though attenuated TGF-β expression could contribute to the decrease in ID1 expression associated with SMOC1 down-regulation, it could not account for the imbalance in ALK5/ALK1 signalling. SPARC, a close homologue of SMOC1 is known to bind to endoglin,[49] a endothelium-specific type III auxiliary receptor for the TGF-β super family that plays a critical role in maintaining cardiovascular homeostasis.[50] Given that SMOC1 and endoglin are both regulated by hypoxia[51] and the pattern of endoglin expression in the postnatal murine retina[52] was similar to that of SMOC1, i.e. elevated at the angiogenic front, two approaches were used to determine whether SMOC1 could elicit its effects via an interaction with endoglin. Using both a proximity ligation assay and co-immunoprecipitation, a direct association of SMOC1 and endoglin could be demonstrated and abrogated by the down-regulation of either protein. Functionally, the phosphorylation of SMAD2, the decrease in α2 integrin expression, and the attenuated migration of endothelial cells that were the result of SMOC1 down-regulation were abrogated by the down-regulation of endoglin. Also, like SMOC1, endoglin is highly expressed in proliferating endothelial cells[36] and is required for the suppression of ALK5 signalling and facilitation of ALK1 signalling.[37] Indeed, endoglin down-regulation was found to counteract ALK5-dependent, TGF-β-induced defects in endothelial cell migration.[53] Thus, SMOC1 may be required for the correct functioning of endoglin. Although SMOC1 and SPARC belong to the same family of matricellular proteins and both bind endoglin, their actions are apparently distinct. While SMOC1 promotes angiogenesis, SPARC has been attributed anti-adhesive and anti-proliferative properties[6,54] and suppresses angiogenesis at least partly by attenuating the expression of VEGF and MMP-7.[55,56] Why two proteins that bind endoglin exert such distinct effects is however currently unclear.

Taken together, the results of the current investigation indicate that miR-223 and SMOC1 are regulators of an angiogenic switch that involves endoglin and a shift in the balance of endothelial cell TGF-β signalling towards ALK1/SMAD1 and SMAD5. It will be interesting to determine to what extent SMOC1 derived from other miR-223 enriched cells, such as monocytes or platelets, contribute to angiogenesis by a similar mechanism.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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