Smad4 deletion in blood vessel endothelial cells promotes ovarian cancer metastasis

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Abstract. SMAD4 is a critical co-smad in signal transduction pathways activated in response to transforming growth factor-β (TGF-β)-related ligands, regulating cell growth and differentiation. The roles played by SMAD4 inactivation in tumors highlighted it as a tumor-suppressor gene. Herein, we report that loss of SMAD4 expression in vascular endothelial cells promotes ovarian cancer invasion. SiRNA transfer of this gene in the HUVEC reduced SMAD4 protein expression and function. Although it reduced the vessel endothelial cell tubule formation *in vitro* and *in vivo*, it did not affect the tumor growth significantly *in vivo*. However, it weakened the barrier integrity in endothelial cells and increased vessel permeability and the ovarian cancer liver metastasis. We documented reduced angiogenesis and increased invasion histologically and by intravital microscopy, and gained mechanistic insight at the messenger and gene level. Finally, we found a negative reciprocal regulation between SMAD4 and FYN. FYN is one of the Src family kinases (SFK), activation of which can cause dissociation of cell-cell junctions and adhesion, resulting in paracellular hypermeability. Upon SMAD4 deletion, we detected high expression levels of FYN in vessel endothelial cells, suggesting the mechanism of the ovarian tumor cells cross the endothelial barrier and transform to an invasive phenotype.

Introduction

SMAD proteins are distributed in the nucleus and cytoplasm. The SMAD4 gene is located on chromosome 18q21 (1), a putative location for other tumor-suppressor genes (2). Loss of Smad4 plays a causal role in initiating squamous cell carcinomas of skin and upper digestive tract as well as adenocarcinomas of gastrointestinal tract, SMAD4 inactivation is associated with a poor prognosis (3). Schwarte-Waldhoff and and Schmiegel (4) used restoration of Smad4 in deficient cancer cells as an impartial approach to reveal the Smad4 tumor suppressor functions. However, stable re-expression of Smad4 in human colon and pancreatic cancer cells potently suppressed tumor growth *in vivo* in nude mice. However, it was not sufficient to suppress tumor cell growth *in vitro*, nor did it restore TGF-β responsiveness. Rather, Smad4 restoration influenced angiogenesis by decreasing expression of vascular endothelial growth factor and increasing expression of thrombospondin-1. These findings suggest that Smad4 not only inhibits the uncontrolled proliferation of epithelial cells, but also mediates tumor promotion predominately through the surrounding stroma (such as endothelial cells) other than the precancerous epithelial cells themselves (5).

Mutations in TGF-β pathway gene SMAD4, have been admitted as genetic causes of a vascular malformation syndrome, hereditary hemorrhagic telangiectasia (HHT) (6-8). Infants and children with a family history of HHT are at risk for sudden and catastrophic intracranial hemorrhage (ICH) (9). However, there is not much research on the role of Smad4 in the cancer blood endothelial cells (BECs).

In this study, we identified that SMAD4 expression is decreased in the vessel ECs of the ovarian cancer. *In vitro* and *in vivo* assays revealed that loss SMAD4 could reduce angiogenesis but increase vessel hyperpermeability and tumor invasion, by modulating the FYN signaling pathway. Taken together, these data highlight the possibility that SMAD4 could be as a therapeutic target in combating ovarian cancer in the future.

Materials and methods

Cell. The human ovarian cancer cell line C13K was a gift from the Department of Obstetrics and Gynecology, Ottawa University, Department of Cell and Molecular Research Center. SKOV3 and A2780 and breast cancer line MDA-MB-231 were purchased from ATCC and cultured according to their guidelines. All the above cell lines used in this study were

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cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum. The cells were used for the experiments within 20 passages. Human umbilical vein endothelial cells (HUVECs) were purchased from Collection of Biological Center, Wuhan University and cultured in endothelial cell medium (ECM; ScienCell) with 5% FBS and endothelial growth medium supplements.

Cell transfection. The Smad4-siRNA sequences used were as follows: GUACUUCAUACCAUGCCGATT and UCGGC AUGGUAUGAAGUACTT. Alternatively, Lipofectamine (Invitrogen)-mediated Smad4-siRNA Oligo transfection was used to knock down Smad4 in HUVECs. Cells were harvested 2 days later for expression analysis or *in vitro* tube formation.

Animals. Female athymic nude (nu/nu) mice (4-week-old) were purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai, China) for studies approved by the Committee on the Ethics of Animal Experiments of Tongji Medical College. The mice were maintained in the accredited animal facility of Tongji Medical College. C13K tumor cells ($3x10^6$) and $1x10^6$ HUVEC were washed, suspended in 50 μl of PBS, and co-injected subcutaneously into nude mice. Tumor volumes were measured using a slide caliper every 3 days according to the formula: volume = (larger diameter) x (smaller diameter)^2 / 2 (10).

Immunohistochemistry. Specimens from normal ovary (14 cases), ovarian carcinoma (19 cases) and normal endometrium (7 cases) were acquired by surgeries as approved by the Ethics Committee of the Medical Faculty of Tongji Medical College (Wuhan, China). The tumor specimens were acquired from patients with cancer who had not undergone preoperative radiotherapy or chemotherapy. Tissue sections were subjected to immunohistochemical (IHC) analysis using the avidin-biotin complex (ABC) Vectastain kit (Zsgb-Bio, Beijing, China) according to the manufacturer's protocol. Anti-human CD34 (Abcam, ab81289), and anti-human SMAD4 (R&D Systems, AF2097) antibodies were used as primary antibodies. Briefly, slides were scanned at low power and the areas with the highest density of CD34-positive vessels were identified. The pathological analyses were done double-blinded.

Western blot analysis. Cells were pre-treated with siRNA as needed. Total proteins were harvested with RIPA buffer. Immunoblotting was performed according to manufacturer's instructions. The relative expression level was quantified using Image-Pro Plus.

In vitro tube formation assay. HUVECs and primary pericytes were mixed and replated to 48-well plates precoated with a thin layer of Matrigel (BD Biosciences) in culture medium containing 5% FCS, and allowed to form tube-like structures for 4-6 h. Measurement was performed as described (11).

In vitro permeability assay. *In vitro* permeability assay were performed as described (12). The concentration of FITC-conjugated dextran (MW40,000, Sigma) was determined with an EnVision fluorescence multiwell plate reader (BD) using a fluorescein filter pair [Ex (l) 480 nm; Em (l)535 nm]. The percentages of control were quantified.

Real-time PCR and microarray analysis. RNA was extracted from HUVECs by TRIzol reagent (Invitrogen) and reverse transcribed by using an mRNA selective PCR kit (Takara). Real-time PCR was performed with Roche LightCycler 2.0 system using a SYBR Green assay. Primers used were as follows: smad4 sense, 5'-CAGGATCAGTAGGTGGAAT AGC-3'; antisense, 5'-TCTTTGATGCTCTGTCTTGGG-3'. INSR sense, 5'-GGAAGTTACGTCTGATTCGAGG-3'; antisense, 5'-TGAGTGATGGTGAGGTTGTG-3'. FYN sense, 5'-ACTATGAAGCACGGACAGAAG-3'; antisense, 5'-TGC TGGGAATGTAACCTGTC-3'. PTPRM sense, 5'-CGATGA GGTGAAGGTGTTAGG-3'; antisense, 5'-ACTGGAAGGT AGCAAACTGG-3'. PTPRJ sense, 5'-CTAGTCCAATTCC TGACCCTTC-3'; antisense, 5'-AGCTTTCACCATCCTCA CTG-3'. VCAN sense, 5'-CACTCTAATCCCTGTCGTAA TGG-3'; antisense, 5'-ATGTCTCGGTATCTTGCTCAC-3'. C CND2 sense, 5'-TGAGGAACAGAAGTGCGAAG-3'; antisense, 5'-TGGTCTCTTTGAGTTTGGAGG-3'. Control-siRNA and Smad4-siRNA HUVECs were subjected to microarray analysis, which was performed using human Gene 1.0 ST array (Affymetrix).

Statistical methods. Data were evaluated using a Student's t-test, two-tailed. $p<0.05$ and $p<0.01$ was considered statistically significant. The error bars on graphs represent the mean ± standard deviation (SD).

Results

Loss of SMAD4 in the blood vessel ECs of the ovarian cancer. To identify the molecular differences between tumor-associated BECs and their normal BEC counterparts, blood vessels were isolated using *in situ* laser capture microdissection and verified by the detection of the mRNA of specific markers. Then, the gene expression profiles of tumor BECs and normal BECs were analyzed using a cDNA microarray as described (13). Since we focused on smad in this study, SMAD4, among the top 10 genes, was chosen for further investigation.

Immunohistochemical analysis of serial sections showed that SMAD4 colocalized with the blood vessel marker CD34. (Fig. 1A). Compared with blood vessels in normal tissues (normal ovary and normal endometrium), the expression of SMAD4 protein in tumor-associated blood vessels was significantly decreased (Fig. 1A). In addition, we showed SMAD4 expression intensity in Fig. 1B). These results suggest that tumor blood vessels might functionally differ from normal blood vessels due to loss of SMAD4 expression.

Effect of SMAD4 deletion in HUVECs in vitro. Research on SMAD4 in ovarian cancer is scarce. Western blotting and immunofluorescence assays were used to detected SMAD4 expression. As shown in Fig. 2A, SMAD4 is expressed universally in 3 ovarian tumor cell lines (SKOV3, A2780 and C13K), human umbilical vein endothelial cells (HUVECs) and one breast cancer cell line (MDA-MB-231) (Fig. 2A). Immunofluorescence analysis of SMAD4 protein are distributed in the nuclear and cytoplasm in HUVEC. In order to simulate the low expression of SMAD4, siRNA was used to interfere the SMAD4 expression in HUVEC, at 48 h, compared

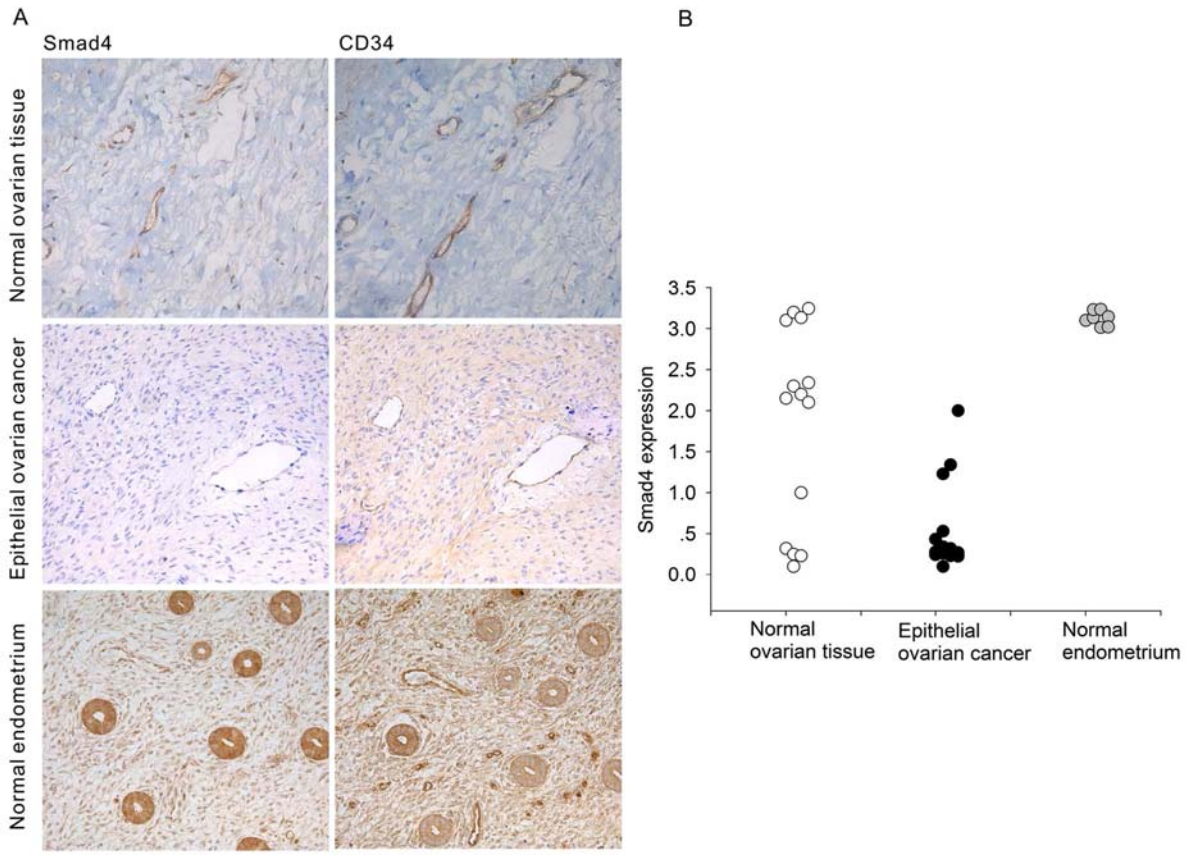

Figure 1. Loss of SMAD4 expression in the blood vessel ECs of ovarian cancer. (A) Immunohistochemical analysis of SMAD4 and the blood vessel marker CD34 in serial sections of human normal ovary, ovarian tumor and normal endometrium specimens. Representative micrographs are shown at x200 magnification. (B) The different intensity of SEMA4C staining in the above three kinds of tissue samples is also shown.

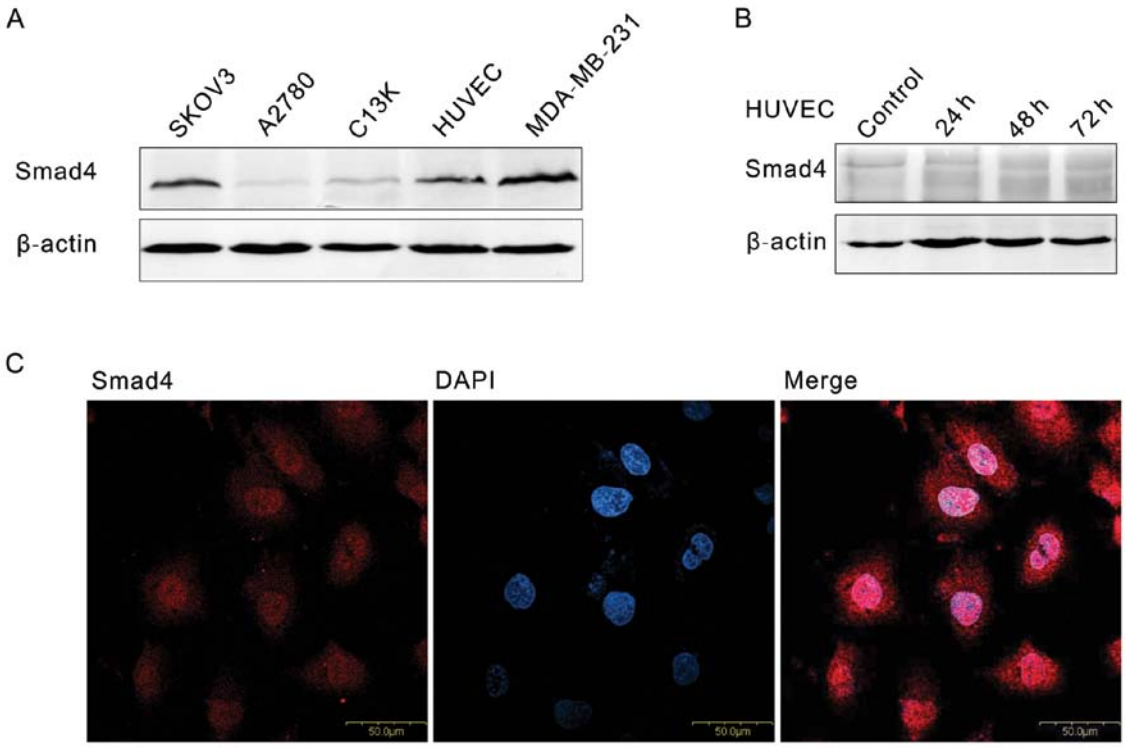

Figure 2. Expression and location of SMAD4 in cell lines. (A) Expression of SMAD4 in 3 ovarian tumor cell lines (SKOV3, A2780 and C13K), human umbilical vein endothelial cells (HUVECs) and one breast cancer cell line (MDA-MB-231). (B) Analysis of Smad4 expression in control- and Smad4-siRNA HUVEC at 24, 48 and 72 h after siSMAD4 transfection. (C) Immunofluorescence analysis of the SMAD4 expression in the HUVECs. Representative images are shown at x400 magnification. Bar, 50 μm.

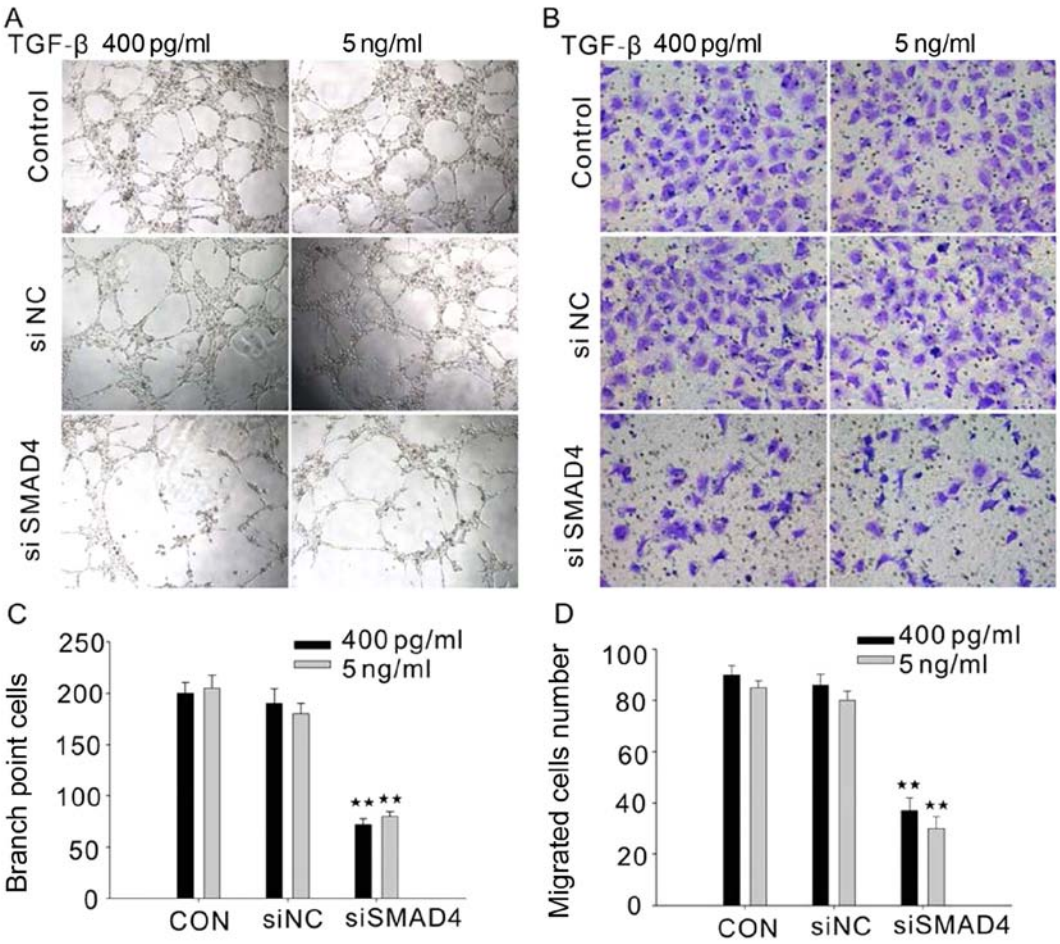

Figure 3. Effect of SMAD4 deletion in HUVECs *in vitro*. (A) *In vitro* tube formation of endothelial cells tansfected with siNC or siSMAD4 under TGF-β (5 ng/ml or 400 pg/ml) treatment. (B) The migration capacity of endothelial cells transfected with siNC or siSMAD4 under TGF-β (5 ng/ml or 400 pg/ml) treatment *in vitro* (**p<0.01).

with control, the SMAD4 expression was decreased 90% (Fig. 2B).

We next investigated the role of SMAD4 in regulation of HUVEC tube formation and migration. TGF-β (5 ng/ml or 400 pg/ml) treated HUVECs with siRNA targeting SMAD4 exhibit a decrease in tube formation and migration (Fig. 3A and B). This result raised the possibility that SMAD4 plays a very important role in angiogenesis.

Effect of SMAD4 deletion in HUVECs in vivo. As SMAD4 deletion decrease the angiogenesis *in vitro*, we next determined whether the effect also occur *in vivo*. We transduced HUVECs with SMAD4-siRNA or control-siRNA and co-inject with C13K subcutaneous in nude mice. Tumor volume was periodically measured, and tumor weight was determined upon dissection at the end of the experiment. Tumor xenografts with SMAD4-deficient HUVECs did not display a significant reduction in volume and tumor burden (Fig. 4A), but they were unexpected associated with a remarkable increase of spontaneous metastatic dissemination to the liver (Fig. 4B). The metastatic potential was quantified by scoring micrometastasis in the liver. Moreover, we found that blood vessels in the tumor with SMAD4-deficient HUVECs were obviously reduced (Fig. 4C). We further investigated whether there was any defect in mural cell coverage. As shown in Fig. 4D, the control tumor vasculature was completely enveloped by mural cells, which were identified by NG2 immunostaining. In comparison, a local smooth muscle cell-coating deficiency was observed in the vessel of Smad4-deficient tumors. These results clearly showed that loss of Smad4 in tumor BECs resulted in defective EC-mural cell contact, which in turn might contribute to decreased mechanical stability, then allow the tumor cells to cross the blood vessel barrier easier and consequently tumor metastasis.

Smad4-deficient HUVECs show decreased barrier function and three-dimensional tube formation in coculture. To explore EC-pericyte interaction *in vitro*, we used an *in vitro* BBB model (13) by coculturing HUVECs with the primary pericytes. When monolayer HUVECs were seeded on the Transwell membrane in tracer permeability assays, there was no apparent difference between siSMAD4 and siNC HUVECs (Fig. 5A and B). Coculture with pericytes reduced the permeability in the control HUVECs to a greater degree than in Smad4-deficient HUVECs, thus, the Smad4-deficient EC barrier supported by pericytes was notably weaker than that of the control.

To further validate the defect in EC-pericyte interaction, we performed an *in vitro* three-dimensional coculture system, in which both ECs and pericytes are morphologically stretched

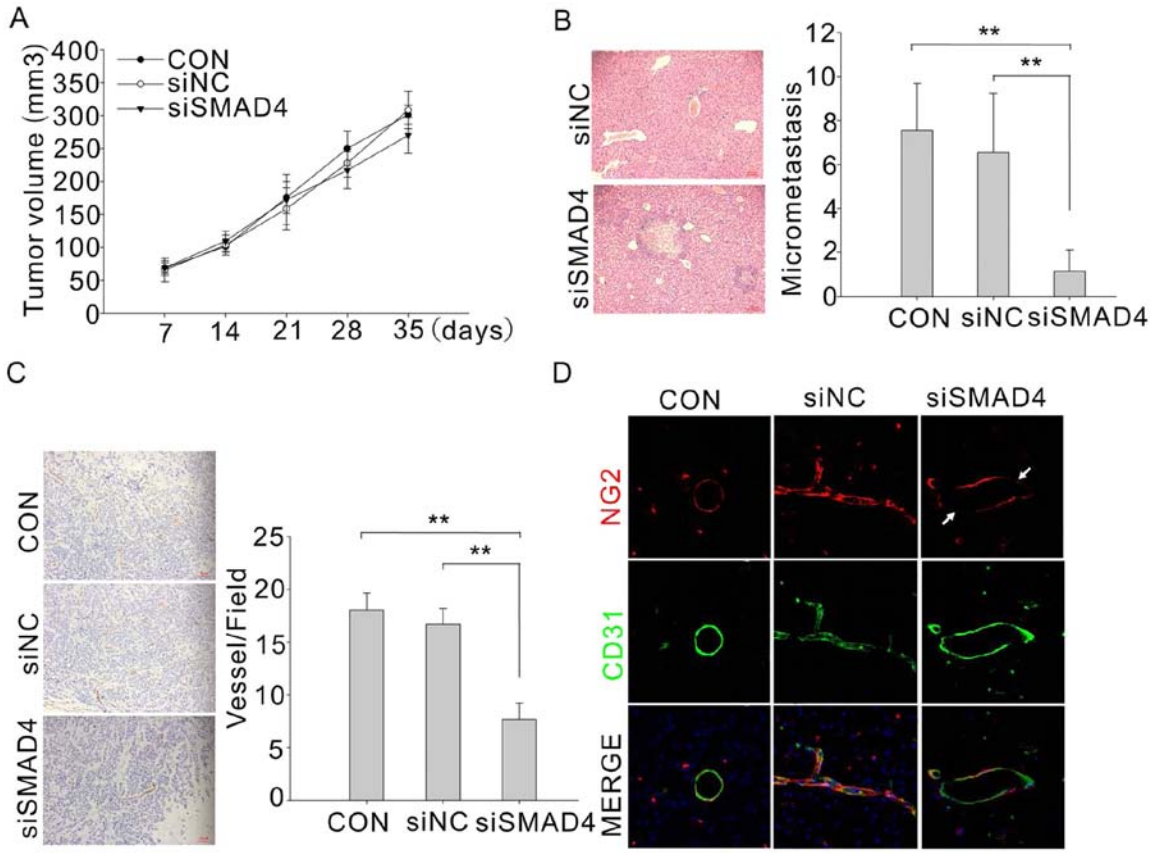

Figure 4. Effect of SMAD4 deletion in HUVECs *in vivo*. (A) Tumor growth of C13K cells with HUVEC tansfected with siNC or siSMAD4. (B) Representative images of liver slices from mice carrying tumor xenografs. Micrometastases present in the liver of mice bearing tumors of comparable size were evaluated as indicated in Materials and methods and scored. (C) CD34 staining in sections from C13K tumors with SMAD4-deficient HUVECs compared to tumors with control HUVECs. (D) NG2 (red) and CD31 (green) double immunostaining on C13K tumor sections demonstrated pericyte coverage defects (arrowheads) in Smad4-deficient HUVECs group. Arrow indicates a rupture of the vessel wall. (**p<0.01).

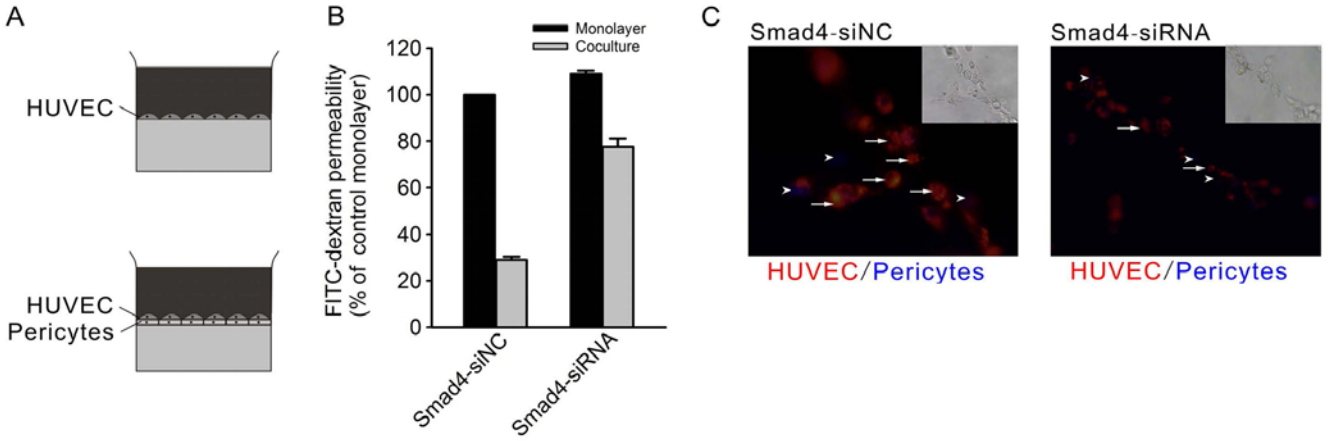

Figure 5. Smad4-deficient HUVECs show decreased barrier function and three-dimensional tube formation in coculture. (A) The *in vitro* BBB model showed how endothelial monolayer or EC-pericyte coculture was seeded on the Transwell membrane. (B) The quantification of Na-F and FITC-dextran permeability showed increased permeability to the tracers in Smad4-deficient HUVECs than in controls when cocultured with the wild-type primary pericytes. (C) Three-dimensional tube formation by coculturing HUVECs (red) and pericytes (blue) on Matrigel. Smad4-siRNA HUVECs showed impaired morphological changes to associating with pericyte to form tube-like structures. White arrows point to HUVECs. White arrowheads indicate pericytes. Inserts are the bright field views of the corresponding panels.

and coordinately formed capillary-like structures (14). With the same primary pericytes in the coculture, Smad4-siRNA HUVECs demonstrated significantly impaired tube-forming capacity compared to the siNC HUVECs. Morphologically, Smad4-siRNA HUVECs showed inefficient elongation and connection with pericytes (Fig. 5C). These results strengthen the opinion that loss of Smad4 in ECs impairs EC-pericyte interaction.

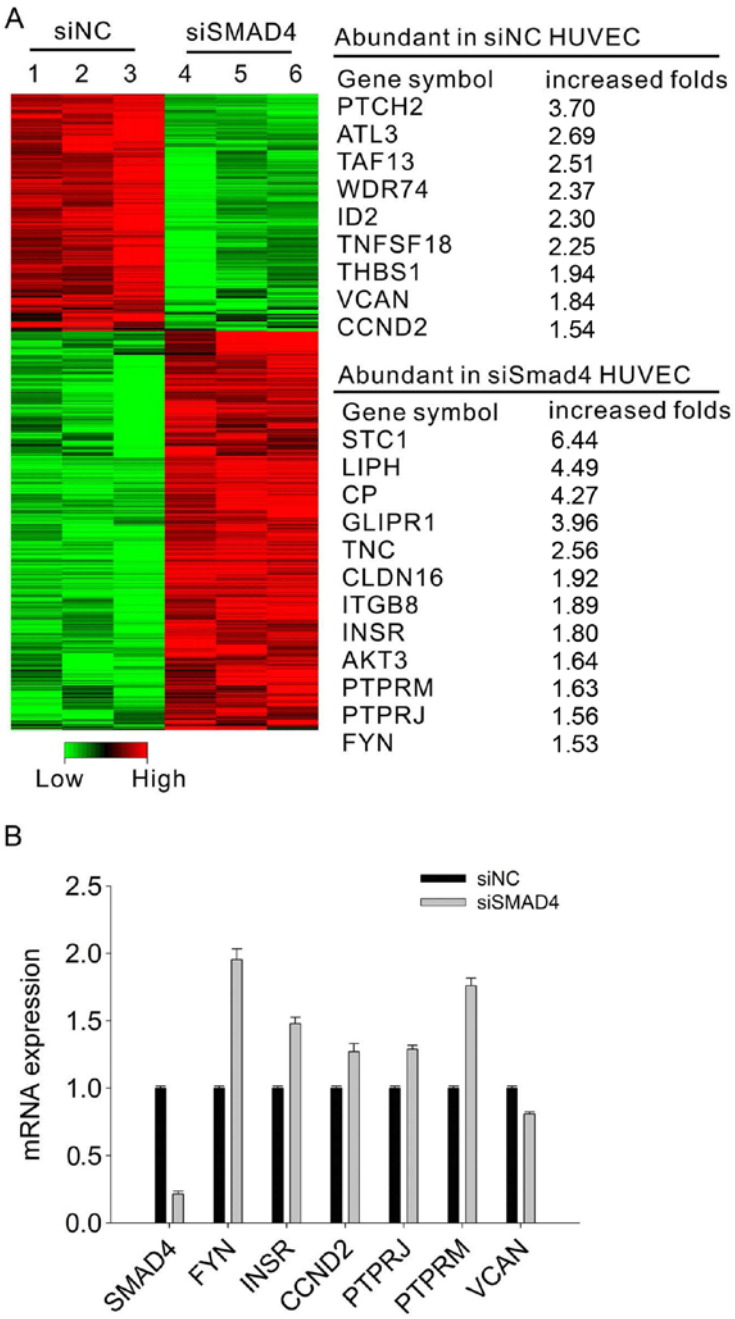

Figure 6. FYN facilitates EC-pericyte interactions mediated by endothelial Smad4. (A) Comparison of the global gene expression profiles associated with siNC versus siSMAD4 tansfected HUVECs (left). Heat maps were developed with the GeneSpring hierarchical clustering algorithm after eliminating all genes for which the difference in the means was less than the SE of the difference of means between groups (right). List of genes and expressed sequence tags overexpressed in HUVEC with siNC or siSMAD4. (B) The expression levels of the indicated genes in HUVECs were detected by real-time RT-PCR.

FYN facilitates EC-pericyte interactions mediated by endothelial Smad4. To identify potential Smad4 target genes that regulate blood vascular integrity in ovarian tumor ECs, a microarray assay was performed to compare the gene expression profiles of HUVECs cells transfected with Smad4-siRNA and control-siRNA. Using GO function enrichment and pathway enrichment analysis, we further verified some expression changes of the genes involving PTCH2, ATL3, PTPR and FYN (Fig. 6A). Of particular interest was the remarkable increase in FYN in Smad4-siRNA HUVECs. We further examined the FYN expression by real-time PCR. As shown in Fig. 6B, the decrease of SMAD4 in the HUVECs induced a remarkably increased FYN expression. These results demonstrated that impaired EC-pericyte interaction in Smad4-deficient ECs might be largely due to the increased FYN expression.

Discussion

The present study reveals an essential role for endothelial Smad4 in the maintenance of ovarian tumor vascular integrity. We show that endothelial Smad4-mediated signaling is required for stabilizing the interaction between vascular ECs and pericytes. Furthermore, we provide a mechanism between

SMAD4 and FYN signaling in maintaining ovarian tumor vascular integrity, which has important implications for the treatment in combating ovarian tumor.

Invading cancer cells could enter the circulation by migrating directly through blood vessel walls (intravasation), which requires the disruption of endothelial junctions. Factors that locally reduce endothelial barrier function, such as transforming growth factor-β (TGFβ) or vascular endothelial growth factor (VEGF), increase the number of cancer cells entering into blood vessels, increase metastasis (15) and contribute to extravasation. While in this study, we found that the SMAD4 expression is reduced in ovarian tumor vessel ECs, which could weaken cell-cell junctions directly.

Pericytes are required for maintaining vascular integrity (16,17), and TGFβ signaling has been identified as an important signal pathway in the differentiation of vascular smooth muscle cells/pericytes at mid-gestation, as revealed by gene knockout research on the signal components, including TGF-β1, Tgfbr2, Alk1, Alk5, endoglin, Smad5, and Smad4 (11,18-22). When an inflammatory agonist (such as TGF-β1) binds to its respective receptor expressed on the endothelial surface, multiple cascades of intracellular signalling reactions are initiated, such as Rho GTPases, MAP kinases and protein kinases (23).

Multiple cascades of intracellular signaling reactions are initiated, such as Rho GTPases, MAP kinases and protein kinases, when an inflammatory agonist binds to its respective receptor expressed on the endothelial surface. Both in the Rho GTPases and MAP kinases signaling activation, the Src protein is playing a pivotal role (23). Also, Src family PTKs (c-Src, Blk, Fgr, Fyn, Hck, Lck, Lyn, Yes and Yrk) have been implicated in the regulation of vascular permeability *in vitro* (24,25) as well as *in vivo* (26,27). Pharmacological inhibition of Src family PTKs has been associated with reduction of vascular permeability in response to several agonists including VEGF (28). Similarly, a role for Fyn has been described for increasing transcellular permeability of microvascular endothelial cells to albumin (29,30).

In this study, we identified that SMAD4 expression is reduced in the vessel ECs of the ovarian cancer. Also, we found that inactivation of SMAD4 could reduce angiogenesis but increase vessel hyperpermeability and tumor invasion *in vitro* and *in vivo*. Use of Gene chip screening of differentially expressed genes, GO function enrichment and pathway enrichment analysis, we discovered that SMAD4 could regulate the FYN expression contrarily. Maybe loss of SMAD4 induced vessel barrier dysfunction by activation of FYN, which will be studied in more detail in the future. Taken together, these data highlight the possibility that SMAD4 could be a therapeutic target in ovarian cancer treatment in the future.

Acknowledgements

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