FGFR1 is critical for the anti-endothelial mesenchymal transition effect of N-acetyl-seryl-aspartyl-lysyl-proline via induction of the MAP4K4 pathway

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Endothelial-to-mesenchymal transition (EndMT) has been shown to contribute to organ fibrogenesis, and we have reported that the anti-EndMT effect of N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) is associated with restoring expression of diabetes-suppressed fibroblast growth factor receptor (FGFR), the key anti-EndMT molecule. FGFR1 is the key inhibitor of EndMT via the suppression of the transforming growth factor β (TGFβ) signaling pathway, and mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4) inhibits integrin β1, a key factor in activating TGFβ signaling and EndMT. Here, we showed that the close proximity between AcSDKP and FGFR1 was essential for the suppression of TGFβ/smado signaling and EndMT associated with MAP4K4 phosphorylation (P-MAP4K4) in endothelial cells. In cultured human dermal microvascular endothelial cells (HMVECs), the anti-EndMT and anti-TGFβ/smado effects of AcSDKP were lost following treatment with a neutralizing FGFR1 antibody (N-FGFR1) or transfection of FRS2 siRNA. The physical interaction between FGFR1 and P-MAP4K4 in HMVECs was confirmed by proximity ligation analysis and an immunoprecipitation assay. AcSDKP induced P-MAP4K4 in HMVECs, which was significantly inhibited by treatment with either N-FGFR1 or FRS2 siRNA. Furthermore, MAP4K4 knockdown using specific siRNAs induced smad3 phosphorylation and EndMT in HMVECs, which was not suppressed by AcSDKP. Streptozotocin-induced diabetic CD-1 mice exhibited suppression of both FGFR1 and P-MAP4K4 expression levels associated with the induction of TGFβ/smado signaling and EndMT in their hearts and kidneys; those were restored by AcSDKP treatment. These data demonstrate that the AcSDKP–FGFR1–MAP4K4 axis has an important role in combating EndMT-associated fibrotic disorders.

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(DPP-4) and integrin β1 induces EndMT by activating TGFβ/smads signaling.32 TGFβ/smads signaling is also inhibited by Misshapen (msn) kinases (the mammalian orthologs of MAP4K4).33 Here, we hypothesize that the AcSDKP–FGFR1–MAP4K4 axis has a key role in the suppression of TGFβ/smads signaling and EndMT in endothelial cells.

Results

The proximity of AcSDKP and FGFR1 is essential for AcSDKP-mediated inhibition of TGFβ/smads signaling pathway in endothelial cells. A duolink in situ proximity ligation assay (PLA) was performed to analyze the proximity between AcSDKP and FGFR1 in cultured human dermal microvascular endothelial cell (HMVEC) (Figure 1a). AcSDKP and FGFR1 displayed close proximity in normal cultured HMVECs (Figure 1a), suggesting that endogenous AcSDKP interacts with FGFR1. Exogenous AcSDKP incubation in endothelial cells further increased the close proximity between AcSDKP and FGFR1 (Figure 1a). In addition, in the presence of a neutralizing FGFR1 antibody (N-FGFR1), which suppresses FGFR1 activity in endothelial cells, the close proximity between AcSDKP and FGFR1 was markedly diminished in HMVECs, even with AcSDKP incubation (Figure 1a). We previously reported that the anti-TGFβ1/smads and anti-kidney fibrosis effects of AcSDKP were associated with the restoration of FGFR phosphorylation (P-FGFR) and FGFR levels.22,34 We also confirmed that AcSDKP significantly inhibited TGFβ2-induced smads3, smad2, TGFβR1, and TGFβR2 levels, and restored TGFβ2-suppressed FGFR1 in endothelial cells (Figures 1b and c and Supplementary Figure S1a). Interferon-γ (IFN-γ) has been confirmed to inhibit FGFR1 protein levels,25 and treatment of HMVECs with AcSDKP was associated with increased FGFR1/P-FGFR1 levels and decreased expression of IFN-γ (Supplementary Figure S1b). Furthermore, Tβ4 restored TGFβ2-suppressed FGFR1 levels, and a POP inhibitor (KYP-2047) abolished the effect of Tβ4 (Supplementary Figure S2a). The bone
morphogenetic protein (BMP) pathway has been associated with EndMT induction. We investigated whether AcSDKP inhibits EndMT by suppressing the BMP pathway, and AcSDKP did not inhibit the BMP-2/BMP-4-induced expression of p-smad1/5/8 in endothelial cells (Supplementary Figure S2b). To further elucidate the significance of AcSDKP on FGFR1 levels and its functional relevance, three AcSDKP mutant peptides, AcSDKA, AcDSPK and AcADKP, were constructed. Western blot analysis revealed that the AcSDKP mutants had no effect on the levels of TGFβ2-induced p-smad3, TGFβR1 and TGFβR2 and suppressed FGFR1 in endothelial cells (Figures 1d and e).

**AcSDKP suppresses TGFβ/smab signaling and EndMT through the FGFR1 pathway.** N-FGFR1 increased the p-smad3 and TGFβR1 protein levels, especially when N-FGFR1 and TGFβ2 were incubated together (Figure 2b). However, in the presence of N-FGFR1, increased p-smad3 and TGFβR1 levels were not suppressed by AcSDKP in endothelial cells (Figure 2b), suggesting that AcSDKP-suppressed TGFβ/smad signaling is dependent on the FGFR1 pathway. Nevertheless, AcSDKP did not bind TGFβR1 or TGFβR2 in endothelial cells (Supplementary Figure S3), likely indicating that there is no distinct interaction between AcSDKP and TGFβRs. FGF2/FGFR1 has been shown to inhibit EndMT induction.25,26

Figure 2: AcSDKP suppresses TGFβ/smab signaling and EndMT through the FGFR1/FRS2 pathway. (a) HMVECs were treated with N-FGFR1 for 48 h, and the FGFR1, TGFβR1 and TGFβR2 protein levels were analyzed by western blot. (b) HMVECs were treated with TGFβ2 in the presence or absence of N-FGFR1 for 15 min with or without AcSDKP preincubation. The p-smad3 and TGFβR1 protein levels were analyzed by western blot. Densitometric analysis of the p-smad3/smad3 and TGFβR1/β-actin levels (n=3) in each group was performed. (c) HMVECs were incubated with either N-FGFR1 in the presence or absence of TGFβ2 for 48 h with or without preincubation with AcSDKP for 2 h or with N-FGFR1 in the presence or absence of TGFβ2 for 48 h with or without 24 h of incubation with FGF2 (50 ng/ml). The CD31, SM22α, FSP1 and α-SMA protein levels were analyzed by western blot. (d) HMVECs were transfected with FRS2 siRNA (100 nM) for 48 h with or without AcSDKP preincubation. The VE-cadherin, FSP1, vimentin, SM22α and p-smad3 levels were analyzed by western blot. (e) HMVECs were treated with N-FGFR1 for 48 h or 15 min in the presence or absence of N-TGFβ(1, 2, 3) (1.0 μg/ml). The CD31, VE-cadherin, SM22α, FSP1, TGFβR1, TGFβR2 and p-smad3 levels were analyzed by western blot. The anti-EndMT effect of AcSDKP via FGFR1 J Li et al Cell Death and Disease
by AcSDKP or FGF2 treatment (Figure 2c and Supplementary Figures S4a and b). Knocking down fibroblast growth factor substrate 2 (FRS2), a key adaptor of FGFR1, using a specific siRNA also induced p-smad3 expression and EndMT (Figure 2d). However, AcSDKP did not suppress FRS2 siRNA-induced p-smad3 and EndMT in endothelial cells (Figure 2d). In addition, a neutralizing TGFβ (1-2-3) antibody (N-TGFβ) completely abolished the N-FGFR1-induced TGFβ/smad signaling and EndMT (Figure 2e). These findings suggest that the anti-TGFβ/smad and anti-EndMT effects of AcSDKP are dependent on the FGFR1/FRS2 signaling pathway.

FGF2/FGFR1 regulates MAP4K4 signaling in endothelial cells. We next investigated the mechanism responsible for decreasing the expression of phosphorylated MAP4K4 (P-MAP4K4) after the FGFR1 pathway was shut down. Immunofluorescence staining revealed that the siRNA-mediated knockdown of FRS2 in endothelial cells significantly reduced the expression of P-MAP4K4 compared with that of the control group (Figure 3a), and TGFβ2-treated cells also showed a similar result (Figure 3a). Western blot analysis was performed to confirm these findings; the expression of P-MAP4K4 in endothelial cells was suppressed by N-FGFR1 and activated by FGF2 (Figures 3b and c). The close proximity between FGFR1 and P-MAP4K4 was observed in normal cultured endothelial cells (Figure 4a), and this close proximity was significantly decreased in the N-FGFR1-treated cells (Figure 4a); TGFβ2 treatment also produced comparable results (Figure 4a). Furthermore, PF-06260933, a potent MAP4K4 inhibitor, significantly suppressed the proximity between FGFR1 and MAP4K4 in endothelial cells (Supplementary Figure S5), suggesting that MAP4K4 phosphorylation is essential for the proximity between FGFR1 and MAP4K4. Immunoprecipitation assays revealed a physical interaction between FGFR1 and P-MAP4K4, and N-FGFR1 decreased this interaction in endothelial cells (Figure 4b).

MAP4K4 signaling is activated by AcSDKP in a FGFR1/FRS2-dependent manner. We next examined whether AcSDKP-activated MAP4K4 signaling in endothelial cells via the FGFR1/FRS2 pathway. Endothelial cells were treated with either N-FGFR1 or FRS2 siRNA in the presence or absence of FGF2 or AcSDKP. In the presence of N-FGFR1, the P-MAP4K4 levels were significantly decreased in endothelial cells and could not be restored by FGF2 (Figure 5a). Similarly, AcSDKP also failed to restore the N-FGFR1-suppressed P-MAP4K4 levels (Figure 5b). Furthermore, in FRS2 siRNA-treated endothelial cells, neither AcSDKP nor FGF2 restored FRS2 siRNA-suppressed P-MAP4K4 levels (Figures 5c and d). These findings suggest that FGFR1 is a key upstream molecule of MAP4K4 signaling in endothelial cells.

MAP4K4 is a key inhibitor of TGFβ/smad signaling and EndMT via suppression of integrin β1 signaling. In endothelial cells, the increased p-smad3 level induced by MAP4K4 siRNA was not suppressed by AcSDKP treatment (Figure 6a). In addition, MAP4K4 siRNA treatment led to decreased expression of the endothelial cell markers VE-cadherin and CD31 and increased expression of the mesenchymal markers FSP1, SM22α and vimentin (Figure 6b), suggesting that MAP4K4 deficiency induces EndMT. However, AcSDKP did not inhibit the MAP4K4 siRNA-induced EndMT (Figure 6b). MAP4K4 has been shown to inhibit the expression of integrin β1 in endothelial cells, and we have previously reported that the interaction between DPP-4 and integrin β1 can induce EndMT. In this study, both TGFβ2 and MAP4K4 siRNA increased the expression of integrin β1 in endothelial cells, especially when TGFβ2 and MAP4K4 siRNA were applied together.
However, in the cells in which MAP4K4 was knocked down, AcSDKP failed to restore the increased levels of integrin β1 (Figure 6c). AcSDKP inhibits diabetes-induced EndMT and restores diabetes-suppressed FGFR1 and P-MAP4K4 expression in mice. AcSDKP restored the high glucose (HG)-induced EndMT in cultured endothelial cells (Supplementary Figure S7). Therefore, to investigate the role of the AcSDKP–FGFR1–MAP4K4 signaling pathway in EndMT induction in vivo, we developed AcSDKP-treated streptozotocin (STZ)-induced diabetic mice. FGFR1 and P-MAP4K4 expression in their cardiac tissue endothelial cells was analyzed by immunofluorescence, and FGFR1 and P-MAP4K4 expression was markedly suppressed in the diabetic hearts compared with the control group (Figure 7a). In addition, AcSDKP treatment restored the diabetes-suppressed endothelial FGFR1 and P-MAP4K4 expression (Figure 7a). We next analyzed endothelial cells in hearts undergoing EndMT, which were recognized by double labeling with CD31/α-SMA or VE-cadherin/SM22α antibodies. The diabetic hearts exhibited more endothelial cells undergoing EndMT compared with the control hearts (Figure 7b), and AcSDKP treatment restored the diabetes-induced EndMT (Figure 7b). When compared with the control mice, the diabetic mice exhibited increased endothelial p-smad3 levels in the hearts, which was inhibited by AcSDKP (Figure 7c). In the hearts, AcSDKP reversed the diabetes-suppressed FGFR1 and P-MAP4K4 levels and the diabetes-induced TGFβ2 levels (Figure 7d). However, the TGFβ1 and TGFβ3 protein levels were not significantly altered (Figure 7d). In kidneys, AcSDKP also restored the diabetes-suppressed FGFR1 and P-MAP4K4 levels and the diabetes-induced TGFβ1 and TGFβ2 expression (Supplementary Figure S6). The TGFβ3 protein level was not significantly altered (Supplementary Figure S6).

Discussion
In our study, we found that AcSDKP inhibited TGFβ/smadr signaling and EndMT in endothelial cells via the FGFR1/
MAP4K4 pathway in vitro and in vivo. In vitro, TGFβ/smad signaling and the associated EndMT were induced by FGFR1 deficiency in a TGFβ2-dependent manner. AcSDKP-activated MAP4K4 signaling through the FGFR1/FRS2 pathway. AcSDKP could not suppress the TGFβ/smad signaling and EndMT induced by MAP4K4 deficiency. In vivo, we also confirmed that AcSDKP restored the expression of FGFR1 and P-MAP4K4 and was associated with TGFβ3/smads expression and EndMT in diabetic hearts and kidneys.

AcSDKP exhibits anti-EndMT and antifibrotic effects in several organ fibrosis models, and EndMT has shown to be induced by TGFβ2 in both a smad-dependent and a smad-independent manner. AcSDKP has also been shown to inhibit TGFβ1-induced p-smad2/3. However, the detailed molecular mechanisms by which AcSDKP inhibits TGFβ/smad signaling and EndMT in endothelial cells are not entirely clear. A recent study confirmed that FGF/FGFR suppressed TGFβ-induced EndMT via the induction of miR-let-7s. Our previous study also demonstrated that AcSDKP restored diabetes-suppressed FGFR and FGFR phosphorylation levels. FGFR1 has been confirmed as a key inhibitor of TGFβ-induced EndMT. Here, we established the FGFR1 signaling-dependent inhibitory effects of AcSDKP on TGFβ/smad signaling and EndMT in HMVECs (Figure 8). AcSDKP
exhibited anti-TGFβ/smад3 and anti-EndMT effects and restored the FGFR1 and P-MAP4K4 levels in diabetic hearts. AcSDKP also suppressed IFN-γ, the potent molecule that inhibited FGFR1. The anti-TGFβ/smад and anti-EndMT effects of AcSDKP were lost when FGFR1 signaling was disrupted.

FGFR1 has a key role in the regulation of cell migration, and the interaction between FGFR1 and integrin β1 is essential for endothelial cell migration. In addition, MAP4K4 promotes endothelial cell migration via inactivation of integrin β1 signaling. Here, we confirmed that FGFR1 was essential for regulation of the MAP4K4 pathway in endothelial cells, as suppression of FGFR1 clearly decreased P-MAP4K4 levels. Conversely, FGF2-induced FGFR1 activation was associated with the induction of P-MAP4K4 (Figure 8).

Kaneko et al. reported that misshapen, the mammalian ortholog of MAP4K4, was a direct suppressor of the TGFβ/smад pathway. Recent studies showed that MAP4K4 deficiency in T cells led to insulin resistance and that MAP4K4 expression was significantly suppressed in type 2 diabetic mice. Despite these important biological roles, little information is known regarding the regulation of MAP4K4 in TGFβ/smад signaling and EndMT induction. Our data demonstrated that AcSDKP-activated P-MAP4K4 expression was dependent on the FGFR1 signaling pathway. In addition, the suppression of MAP4K4 in endothelial cells significantly induced TGFβ/smад signaling and EndMT, even in the presence of AcSDKP (Figure 8). In vivo, we demonstrated that AcSDKP reversed the diabetes-suppressed FGFR1 and P-MAP4K4 levels associated with the induction of TGFβ/smад signaling and EndMT in endothelial cells in hearts and kidneys.

Figure 7  AcSDKP inhibits TGFβ/smад signaling and EndMT and restores the FGFR1 and P-MAP4K4 levels in diabetic hearts. (a) Immunofluorescence microscopy analysis of CD31/FGFR1 and CD31/P-MAP4K4 in the heart tissues from each group of mice. The scale bar is 60 μm in each panel. The CD31 and FGFR1 double-labeled cells and the CD31 and P-MAP4K4 double-labeled cells in each visual field were assessed by fluorescence microscopy and quantified. For each section, images from six different fields of view at x 400 magnification were evaluated. (b and c) Immunofluorescence microscopy analysis of CD31/α-SMA, VE-cadherin/SM22α and CD31/β-smad3 expression levels in the heart tissues from each group of mice. The scale bar is 60 μm in each panel. The CD31 and α-SMA double-labeled cells, the VE-cadherin and SM22α double-labeled cells and the CD31 and p-smad3 double-labeled cells in each visual field were analyzed by fluorescence microscopy and quantified. For each section, images from six different fields of view at x 400 magnification were evaluated. Four mice from each group were analyzed. (d) Western blot analysis of the FGFR1, P-MAP4K4, TGFβ1, TGFβ2 and TGFβ3 levels in cardiac tissues. A representative blot from four independent experiments was shown. The densitometric analysis of western blot data was presented (n = 4). The diabetic mice are abbreviated as DM in the figure.
Figure 8 Schematic of the AcSDKP/FGFR1/MAP4K4 pathway suppression of TGFβ/smad signaling and EndMT: In endothelial cells, the close proximity between AcSDKP and FGFR1 increased FGFR1 and induced its phosphorylation levels. Interacting with co-factor FRS2, FGFR1 recruited MAP4K4 and induced its phosphorylation. Subsequently, p-MAP4K4 suppressed integrin/1 (integrin/1 should be localized on the cell surface interacted with some of α-integrins). Integrin/1 was a potent activator of TGF-β signaling and also EndMT. Therefore, AcSDKP could inhibit EndMT through FGFR1-MAP4K4-dependent manner.

MAP4K4 has been shown to suppress integrin β1 signaling in endothelial cells and integrin β1 is associated with TGFβ signaling and EndMT induction in endothelial cells. In our study, suppression of MAP4K4 increased the integrin β1 levels in endothelial cells, and AcSDKP had no effect on its levels in MAP4K4-deficient cells (Figure 8). These data confirmed that MAP4K4 is a key downstream molecule for the anti-EndMT effect of AcSDKP.

Our results confirmed the functional interactions among AcSDKP, FGFR1 and MAP4K4 in endothelial cells. The close proximity between AcSDKP and FGFR1 inhibited the EndMT associated with TGFβ/smad signaling by activating the MAP4K4 pathway. In addition, AcSDKP restored both diabetes-suppressed FGFR1 and P-MAP4K4 levels and induced TGFβ/smad signaling and EndMT in heart and kidney. These findings reveal an AcSDKP–FGFR1–MAP4K4 signaling axis, offering new insights into endothelial cell biology and a potential target for future studies of EndMT-associated organ fibrosis.

Materials and Methods

Reagents. The AcSDKP peptide was a gift from Asabio Bio Technology (Osaka, Japan). The AcSDKA (100 nmol, SQ14640), AcDSPK (100 nmol, SQ14641) and AcDKDP (100 nmol, SQ14639) peptides were purchased from Scrum Inc. (Tokyo, Japan). The following antibodies were purchased from Abcam (Cambridge, UK): mouse monoclonal anti-FGFR1 (1:500, ab8232), rabbit polyclonal anti-integrin β1 (1:500, ab115146), mouse monoclonal anti-vimentin (1:2000, ab9878), rabbit polyclonal anti-α-SMA (1:1000, ab5694), rabbit monoclonal anti-IFN-γ (1:1000, ab133566), rabbit polyclonal anti-TGFβR1 (1:1000, ab662103) and rabbit polyclonal anti-TGFβR2 (1:500, ab15337). The human BMP-4 peptide (1 μg/ml, ab40140) and the human TGFβ (1 μg/ml, ab42293) were also purchased from Abcam. The mouse monoclonal anti-human CD31 (1:1000, AF3628), human neutralizing FGFR1 (1:500, MAB765) and neutralizing TGFβ1 (1:2-3) (1:500, MAB1835) antibodies were purchased from R&D Systems (Minneapolis, MN, USA). A rabbit polyclonal anti-phospho-smad3 (s423 and s425) (1:1000, 600-401-919) antibody was purchased from Rockland Immunchemicals (Gilbertsville, PA, USA). The mouse monoclonal anti-β-actin (1:100 000, A2228), rabbit anti-TGFβR1 (1:1500, SAB4502954) and rabbit anti-TGFβR2 (1:1500, SAB4502958) antibodies, as well as FGFR2 human recombinant (50 μg/ml, SRP4037), KYP-2047 (1 μg/ml, SML0208) and PF-06260933 (1 μmol, PZ-0272) were obtained from Sigma (St. Louis, MO, USA). The rabbit polyclonal anti-phospho-HGK/MAP4K4 P5Ser801 antibody (1:500, PA5-12874) and the rabbit anti-SMAD2 (1:500, #51-1300) antibody were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The rabbit polyclonal anti-HGK/MAP4K4 (1:500, NBPI-58853), rabbit polyclonal anti-SM22α (1:1000, NBPI-33003) and rabbit monoclonal anti-VE-cadherin (1:1000, NBPI-34374) antibodies, along with the recombinant human BMP-2 protein (1 μg/ml, NBPI-98923) were obtained from Novus Biologicals (Littleton, CO, USA). The rabbit polyclonal anti-S100A4 (also known as FSP1) (1:200, PRB-497P), rabbit polyclonal anti-FRS2 (1:1000, sc-8318) and goat polyclonal anti-p-smad1/5/8 (1:1000, sc-12353) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The rabbit polyclonal anti-AcSDKP (1:1000, A03881) antibody was purchased from Biocompare (South, San Francisco, CA, USA). The rabbit polyclonal anti-smad3 (1:1000, #9513), rabbit polyclonal anti-p-smad2 (1:400, #31085) and rabbit anti-phospho-FGFR1 (1:500, #3471) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). The TGFβ2 antibody (1:1000, GTX15539) was purchased from Gene Tex (Alton Pkwy, Irvine, CA, USA).

Western blot analysis. Proteins were harvested using RIPA lysis buffer (lysis buffer, PMSF, protease inhibitor cocktail and sodium orthovanadate, which was purchased from Santa Cruz Biotechnology). The protein lysates were boiled in SDS sample buffer at 100 °C for 6 min, separated on SDS-polyacrylamide gels, and then transferred onto PVDF membranes (Pall Corporation, Pensacola, FL, USA) using the semi-dry method. After blocking, the membranes were incubated overnight with primary antibodies at 4 °C, followed by the corresponding peroxidase-conjugated secondary antibody for 1 h at room temperature. The blots were developed with an ECL Plus Western Blotting Detection System (Amersham). The proteins were quantified by densitometry using ImageJ software. The data were analyzed by Student’s t-test.
Immunoprecipitation. HMVECs were treated with either N-FGFRI or TGFβ2. After 48 h, the cells were harvested using the RIPA lysis buffer system and centrifuged at 14,000×g for 15 min at 4 °C. The supernatant was then transferred to a new tube, washed twice with protein A solution (Cell Signaling Technology), and incubated with primary antibody overnight at 4 °C. Protein A was added again, and incubated for 2 h at 4 °C. Next, the solution was centrifuged, and the supernatant was discarded. The pellets were washed three times with RIPA lysis buffer. After the supernatant was discarded, 2× SDS sample buffer was added to the pellets, and the mixture was boiled for 5 min to elute the captured proteins for western blotting.

Immunofluorescence for cell culture. The treated HMVECs were cultured on eight-well culture slides for 48 h. The cells were then fixed with 100% methanol for 10 min at −20 °C and acetone for 1 min at −20 °C. After blocking with 2% BSA/PBS for 30 min at room temperature, the cells were incubated with primary antibody for 1 h. After being washed with PBS, the slides were incubated with the corresponding secondary antibodies for 30 min. The cells were then extensively washed three times with PBS and mounted with mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). The images were analyzed by fluorescence microscopy (Axio Vert.A1, Carl Zeiss Microscopy GmbH, Jena, Germany).

Immunostaining analysis of mouse tissue. Frozen heart sections were fixed with acetone for 10 min at −20 °C. The sections were blocked with 2% BSA/PBS for 30 min at room temperature and incubated with primary antibodies against CD31/FGFRI, CD31/P-MAPK44, CD31/α-SMA, VE-cadherin/Smad2 or 2α and CD31/p- Smad3 for 1 h. The sections were then incubated with the corresponding secondary antibodies for 30 min at room temperature and mounted in mounting medium containing DAPI (Vector Laboratories). The images were analyzed by fluorescence microscopy (Axio Vert.A1, Carl Zeiss Microscopy GmbH).

Animal experiments. Control, STZ-treated CD-1 and AcSDKP-treated STZ mice were prepared based on our previous report.22 Briefly, 8-week-old CD-1 mice were injected with STZ (200 mg/kg). Sixteen weeks after the induction of diabetes, FGFRI and EndMT were examined. 8-10 mice were examined for each group. The treated HMVECs were cultured and subjected to immunofluorescence analysis. The images were analyzed by fluorescence microscopy (Axio Vert.A1, Carl Zeiss Microscopy GmbH).

Statistical analysis. The data are expressed as the mean ± S.E.M. One-way ANOVA followed by the Tukey's multiple comparison test (statistical significance was defined as P < 0.05) was used for statistical analysis using GraphPad Prism software (Ver 5.0f, La Jolla, Canada).

Conflict of Interest
The authors declare no conflict of interest.

Author contributions
JL performed all the in vitro experiments, as well as some animal experiments, and participated in writing the manuscript. SS helped perform the animal experiments and supervised the in vitro experiments. SPS supervised the experiments, provided intellectual input, and wrote the manuscript. DK provided intellectual input.

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