FGF signaling contributes to atherosclerosis by enhancing the inflammatory response in vascular smooth muscle cells

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Received June 14, 2018; Accepted March 7, 2019

DOI: 10.3892/mmr.2019.10249

Abstract. The contractile to synthetic phenotypic switching of vascular smooth muscle cells (VSMCs) in response to fibroblast growth factor (FGF) has been previously described. However, the role of the inflammatory response induced by FGF signaling in VSMCs and its occurrence in atherosclerosis remains unclear. In the present study, FGF signaling promoted a contractile to secretory phenotypic transition in VSMCs. VSMCs (primary human aortic smooth muscle cells) treated with FGF exhibited a decrease in the protein expression levels of factors involved in contractility and the secretion of various chemokines was increased, as assessed by reverse transcription-quantitative PCR and ELISA. Additionally, inhibition of FGF signaling by silencing FGF receptor substrate 2 (FRS2) decreased the protein expression levels of various chemokines. Furthermore, VSMCs in the medial layers of arteries from apolipoprotein E-deficient mice and human atherosclerotic samples exhibited an increase in FGF signaling that was identified to be associated with an increase in the protein expression levels of pro-inflammatory molecules, including C-C motif chemokine ligand 2, C-X-C motif chemokine ligand (CXCL) 9, CXCL10 and CXCL11, compared with wild-type mice and healthy control samples, respectively. The present results suggested that FGF signaling induced dedifferentiation of contractile VSMCs and the transition to a secretory phenotype, which may be involved in the progression of atherosclerosis. Collectively, the present results suggested that the FGF signaling pathway may represent a novel target for the treatment of atherosclerosis.

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Key words: fibroblast growth factor signaling, chemokines, vascular smooth muscle cells, inflammatory response, atherosclerosis

Introduction

Atherosclerosis is the most common cause of cardiovascular diseases (1). The processes involved in atherosclerosis are complex and include endothelial cell activation, phenotypic switching in vascular smooth muscle cells (VSMCs) and leukocyte infiltration (2). However, the role of VSMCs in atherosclerosis remains unclear. Mature VSMCs in the medial layer of arteries exhibit a contractile phenotype and express multiple contractile proteins (3). In the context of atherosclerosis, medial VSMCs undergo a phenotypic switch to a synthetic state (3). VSMCs may lose contractile markers and acquire the ability to proliferate and synthesize various components involved in extracellular matrix deposition, which is involved in the growth of atherosclerotic plaques following the migration of VSMCs in the neointima (4). Under stress conditions, including organ transplant rejection, synthetic VSMCs are involved in extracellular matrix deposition and express a variety of chemokines that recruit alloreactive T cells to the vascular wall (5-7). Therefore, medial VSMCs may accelerate the atherosclerotic process by releasing chemokines and promoting leukocyte infiltration.

The phenotypic plasticity of VSMCs is regulated by a number of signaling pathways (8). Fibroblast growth factor (FGF) is one of the growth factors released by VSMCs and exhibits the ability to induce VSMC dedifferentiation and proliferation. FGF receptor substrate 2 (FRS2) is a scaffold protein interacting with FGF receptors (FGFRs) that mediates multiple signaling pathways to the activated FGFR (9). Following the activation of the FGF signaling pathway, VSMCs undergo a phenotypic switch from a contractile to a synthetic form (10). Activated FGF signaling inhibits the expression level of transforming growth factor β receptor (TGFBR) 1 expression via the microRNA let-7 (11-13). Following activation of the FGF signaling pathway, the phosphorylation of SMAD2/3 decreases and transforming growth factor β (TGFβ) signaling is repressed (14,15). TGFβ was previously demonstrated to maintain VSMCs in a quiescent state and contractile phenotype (16). In the present study, FGF signaling was hypothesized to promote a secretory phenotype in VSMC, promoting the expression of factors involved in extracellular matrix deposition and pro-inflammatory factors.

In the present study, human VSMCs, and mouse and human atherosclerotic samples were used to investigate the role of

FGF signaling in VSMCs. FGF was identified to promote a phenotypic transition in VSMCs, inducing VSMCs to secrete various chemokines previously identified to be involved in atherosclerosis, including C-C motif chemokine ligand 2 (CCL2), C-X-C motif chemokine ligand (CXCL) 9, CXCL10 and CXCL11 (5-7). In the present study, medial VSMCs were identified to secrete various chemokines in mouse and human atherosclerotic samples, and exhibited an increase in FGF signaling. Collectively, the present results suggested that FGF signaling induced the release of chemokines by VSMC that may be involved in leukocyte infiltration and plaque formation in the atherosclerotic process.

Materials and methods

Cells and short hairpin RNA (shRNA). Primary human aortic smooth muscle cells (HASMCs) were purchased from Lonza Group, Ltd. (Basel, Switzerland) and cultured in Smooth Muscle Cell Growth Medium-2 supplemented with 5% FBS (Lonza Group, Ltd.). Following 5-8 passages, HASMCs were used for further experiments. For reverse transcription-quantitative PCR (RT-qPCR) analysis, cells were serum-deprived in 0.5% FBS for 48 h prior to treatment with FGF1 (50 ng/ml, BioLegend, Inc., San Diego, CA, USA). Untreated cells were used as control. For FGF signaling inhibition, HASMCs were washed with Hank's balanced salt solution and transfected with a lentivirus encoding an shRNA (5'-CTCTAAATGGCT ACCATAATA-3'; Open Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) targeting FRS2 for 6 h according to the manufacturer's protocol. To construct FRS2α shRNA lentivirus, 10 μg pLVX-IRES-puro (Addgene, Inc.) carrying the FRS2α cDNA expression cassette, 2.5 μg of RSV-REV plasmid (Addgene, Inc.), 5 μg pMDLg/PRRE vector and 3 μg pMD.2G vector (Addgene, Inc.) were cotransfected into 293T cells using FuGENE 6 (Promega Corporation, Madison, WI, USA). The medium was harvested 48 h later and HASMCs were treated with the resulting medium. An empty pLVX-IRES-puro vector was used as control. HASMCs were grown at 37˚C and 5% CO_2 in smooth muscle cell growth medium-2 (Lonza Group, Ltd., Basel, Switzerland). 48 h after shRNA transfection, HASMCs were used for subsequent experiments.

RNA extraction and analysis. The total RNA was extracted from cultured cells using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Tri Reagent was used (Molecular Research Center, Inc., Cincinnati, OH, USA) to homogenize the cells. RNA samples were treated with deoxyribonuclease I (DNase I; Ambion; Thermo Fisher Scientific, Inc.) and were reverse-transcribed using random hexamers and oligo-dT using the Multiscribe RT system (TaqMan RT reagents; Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The RT reaction was performed at 25˚C for 5 min, followed by incubations at 46˚C for 20 min and at 95˚C for 1 min. The RT-qPCR was performed in 20 μl total volume in duplicate using cDNA samples derived from total RNA. TaqMan Universal PCR Master Mix, primers and TaqMan fluorescent probes were purchased from the Assays-by-Design service (Applied Biosystems; Thermo Fisher Scientific, Inc.). Thermocycling conditions were as follows: Uracil-DNA glycosylase activation at 50˚C for 2 min, initial denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. Using the AmpliTaq Gold polymerase (Thermo Fisher Scientific, Inc.). Samples were analyzed using CFX manager 3.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Non-retrotranscribed RNA samples were used as negative control. The expression levels of the target genes were normalized to GAPDH and quantified using the $2^{-\Delta\Delta Cq}$ method (17). The primers used in the present study were: Actin α 2, smooth muscle (ACTA2; cat. no. Hs05005341_m1), calponin 1 (CNN1; cat. no. Hs00154543_m1), TGFβR2 (cat. no. Hs00234253_m1), CCL2 (cat. nos. Hs00234140_m1 and Mm00441242_m1), CXCL9 (cat. nos. Hs00171065_m1 and Mm00434946_m1), CXCL10 (cat. nos. Hs00171042_m1 and Mm00445235_m1), CXCL11 (cat. nos. Hs00171138_m1 and Mm00444662_m1) and GAPDH (cat. nos. Hs02786624_g1 and Mm99999915_g1).

ELISA. Chemokine concentrations of human CCL2 (cat. no. DCP00), CXCL9 (cat. no. DCX900), CXCL10 (cat. no. DIP100) and CXCL11 (cat. no. DCX110) from cultured VSMCs were determined by ELISA assay (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. Samples and standards were added to the wells and plates were incubated for 2 h at room temperature. Detection solution was added to each well and the plates were further incubated for 2 h at room temperature. Substrate solution was added to each well and plates were incubated for 30 min at room temperature. Stop solution was added to each well and the optical density was detected using a Fusion® plate reader (PerkinElmer, Inc., Waltham, MA, USA) after 30 min.

Animal studies. In total, 16 male C57BL/6J mice (WT; age, 8-12 weeks; body weight, 25 g) and apolipoprotein E (ApoE)-deficient mice ($ApoE^{-/-}$; age, 8-12 weeks; body weight, 25 g) were purchased from The Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). Mice were housed under a 12-h light/dark cycle and maintained at a temperature of 30-34˚C with 30-70% relative humidity. The WT mice were fed with standard rodent chow and the $ApoE^{-/-}$ mice were fed with a high-fat diet (normal chow supplemented with 1.25% cholesterol and 40% kcal fat) for 4 months. Each group consisted of eight mice. To harvest the aortic arteries, mice were anesthetized and perfused through the left ventricle sequentially with 0.9% sodium chloride. Mouse brachiocephalic arteries were removed under a dissection microscope and preserved in 10% paraformaldehyde at 4˚C prior to embedding in optimum cutting temperature (OCT) compound. Samples were cut into 5-μm thick transverse sections for immunofluorescence, and 10-μm thick transverse sections were cut for laser-capture microdissection and RT-qPCR analysis. Animal experiments were performed according to published guidelines (5), and the experimental procedures were approved by The Ethics Committee of The First Hospital of China Medical University (Shenyang, China).

Artery donors. The Ethics Committee of The First Hospital of China Medical University approved the research protocols and the experiments involving human subjects. All donors provided written informed consent. The families of the

Table I. Clinical characteristics of patients with atherosclerosis and healthy subjects.

Characteristic	Healthy subjects (n=6)	Patients with atherosclerosis (n=6)
Age (years)	38.9±4.6	62.2±12.6
Female to male ratio	1:1	1:5
Hyperlipidemia	0 (0%)	5 (83.3%)
Current smoker	0 (0%)	2 (33.3%)

patients provided informed consent for the use of samples from deceased organ donors. Clinical characteristics are listed in Table I. Only patients >18 years old were enrolled in the present study. Patients with cardiomyopathy and aortic aneurism were excluded from the present study. In total, six patients (age, 62.2±12.6 years; 1 female, 5 males) with coronary atherosclerosis assessed by histological examination and six healthy subjects (age, 38.9±4.6 years; 3 females, 3 males) were enrolled in the present study. Human coronary arteries were collected from explanted hearts of transplant recipients or deceased organ donors. The samples were collected in The First Hospital of China Medical University between January 2008 and December 2018.

Immunofluorescence. Human coronary arteries or mouse brachiocephalic arteries were flushed with PBS, fixed in 10% paraformaldehyde at 4˚C overnight, embedded in OCT blocks, and cut into 5-μm thick sections at -20˚C. Sections were incubated with primary antibodies at 4˚C overnight, washed with TBS three times, and incubated with Alexa Fluor®-conjugated secondary antibodies at room temperature for 1 h. The following primary antibodies were used: Mouse anti-smooth muscle actin (SMA; 1:200; cat. no. ab7817; Abcam, Cambridge, UK), rabbit anti-human/mouse phosphorylated (p-)FGFR1 (1:100; cat. no. ab59194; Abcam) and rabbit anti-human/mouse TGFβR2 (1:100; cat. no. ab216483; Abcam). The following secondary antibodies were used: Alexa Fluor® 488 donkey anti-mouse IgG (1:200; cat. no. A21202; Invitrogen; Thermo Fisher Scientific, Inc.) and Alexa Fluor® 594 donkey anti-rabbit IgG (1:200; cat. no. A21207; Thermo Fisher Scientific, Inc.). Five sections from each graft and four fields of view for each section were analyzed. The nuclei of positive cells were counted using an epifluorescence microscope under high magnification (magnification, x400).

Laser-capture microdissection. Human coronary arteries or mouse aortic arteries were flushed with PBS without fixation, embedded in OCT blocks, cut into 10-μm thick sections at -20˚C and placed on nuclease-free, membrane-covered frame slides. The medial layer of the aortic wall, primarily consisting of VSMCs, was cut using a Pixcell laser-capture epifluorescence microscope (magnification, 100x; Arcturus; Thermo Fisher Scientific, Inc.) with a laser power setting of 38 mW, pulse duration of 1 msec, and a 10 μm spot size. The cellular material was captured using CapSure LCM caps (Thermo Fisher Scientific, Inc.) and digested in Nanoprep lysis buffer (Agilent Technologies, Inc., Santa Clara, CA, USA). The DNA content in each sample was quantified using the Picogreen fluorometric assay system (Molecular Probes; Thermo Fisher Scientific, Inc.). The total RNA was

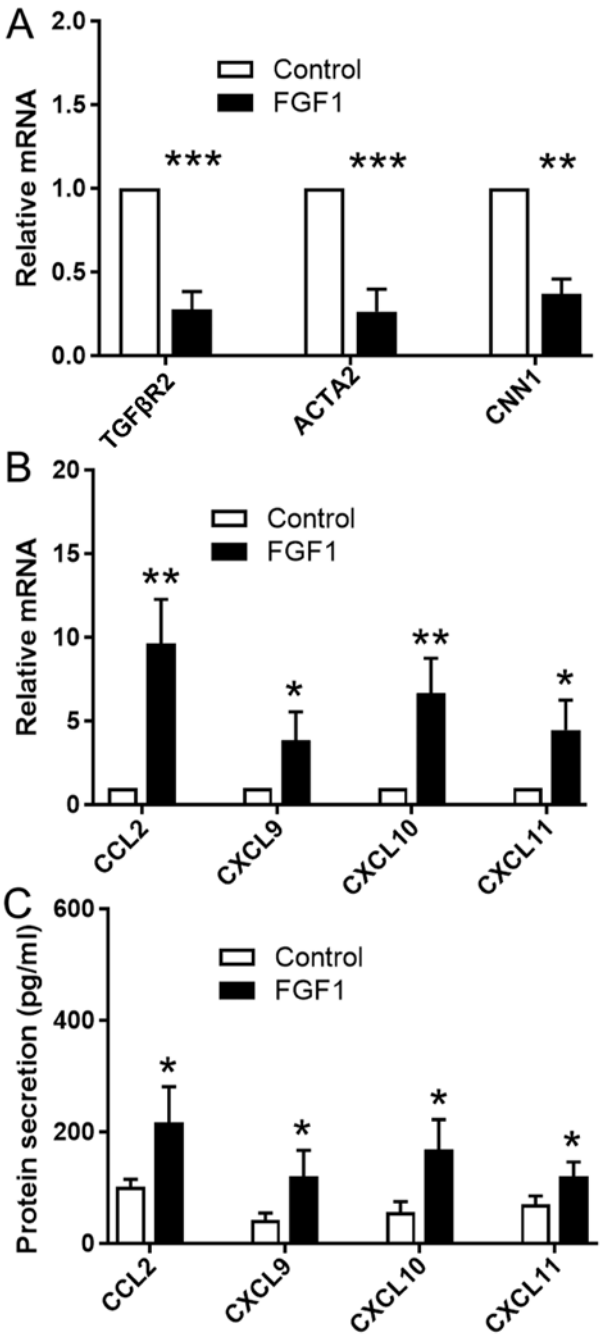

Figure 1. FGF signaling downregulates the expression level of TGFβR2 and upregulates the expression levels of various chemokines in VSMCs. Human aortic smooth muscle cells were treated with or without FGF1 (50 ng/ml). Reverse transcription-quantitative polymerase chain reaction was used to assess the mRNA expression levels of (A) TGFβR2, ACTA2, CNN1 and (B) various chemokines. GAPDH was used as the internal control. Data are presented as fold changes in treated cells relative to untreated controls. (C) Quantification of chemokines levels secreted by VSMCs by ELISA. Experiments were repeated three times. Data are presented as the mean ± standard error. Statistical significance was determined by unpaired two-tailed Student's t-test. *P<0.05, **P<0.01, ***P<0.001 vs. untreated cells. VSMCs, vascular smooth muscle cells; FGF1, fibroblast growth factor 1; TGFβR2, transforming growth factor β receptor 2; ACTA2, actin α 2, smooth muscle; CNN1, calponin 1; CCL2, C-C motif chemokine ligand 2; CXCL, C-X-C motif chemokine ligand.

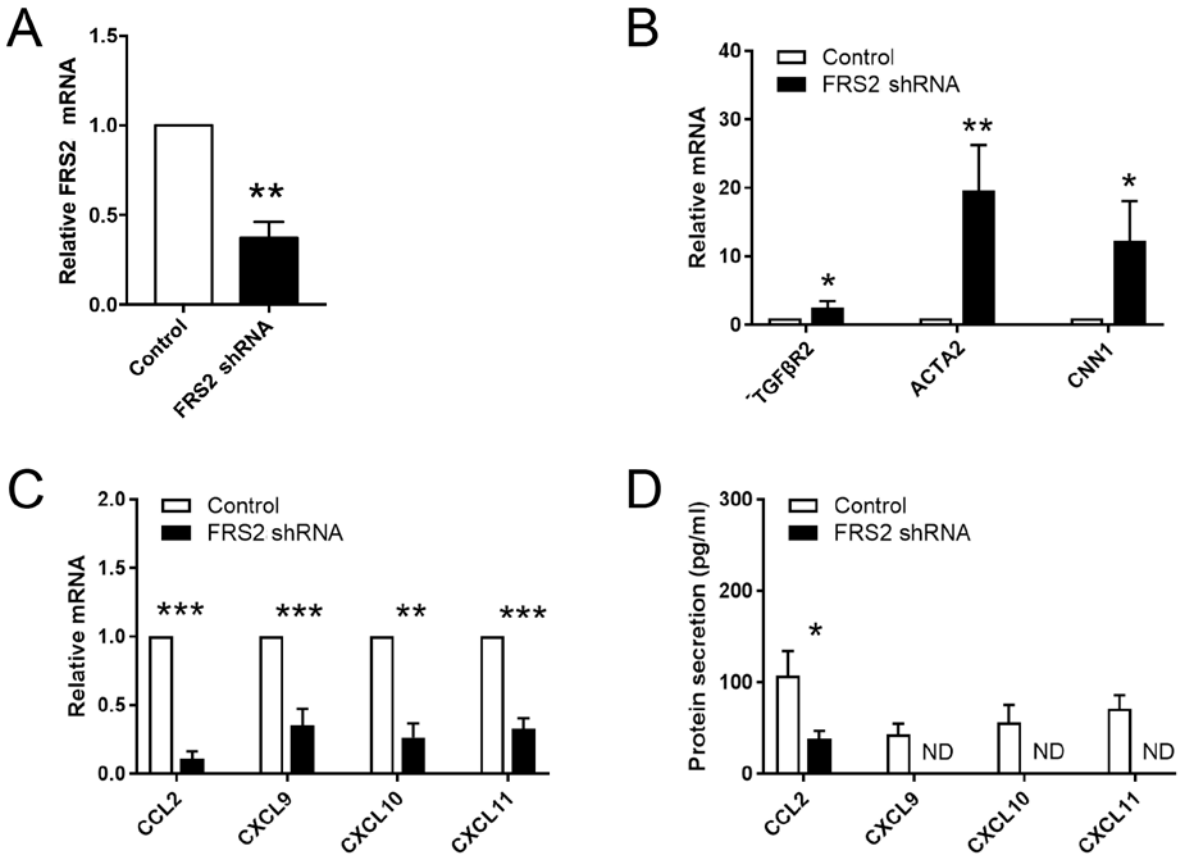

Figure 2. Inhibition of FGF signaling upregulates TGFβR2 and downregulates chemokines in VSMCs. Human artery smooth muscle cells were transfected with FRS2 to inhibit FGF signaling. (A) Efficiency of shRNA transfection in VSMCs was measured by RT-qPCR. RT-qPCR was used to assess the expression levels of (B) TGFβR2 and (C) various chemokines. GAPDH was used as the internal control. Data are presented as fold changes in transfected cells relative to untreated controls. (D) Protein levels of chemokines secreted by VSMCs were determined by ELISA. Experiments were repeated three times. Data are presented as the mean ± standard error. Statistical significance was determined by unpaired two-tailed Student's t-test. *P<0.05, **P<0.01, ***P<0.001 vs. empty vector control. FGF, fibroblast growth factor; VSMCs, vascular smooth muscle cells; RT-qPCR, reverse transcription-quantitative PCR; FRS2, fibroblast growth factor receptor substrate 2; shRNA, short hairpin RNA; TGFβR2, transforming growth factor β receptor 2; ACTA2, Actin α 2, smooth muscle; CNN1, calponin 1; CCL2, C-C motif chemokine ligand 2; CXCL, C-X-C motif chemokine ligand; ND, not detectable.

isolated using the PicoPure RNA isolation kit (Thermo Fisher Scientific, Inc.). RNA samples were treated with DNase I (Ambion; Thermo Fisher Scientific, Inc.) and were reverse-transcribed using random hexamers and oligo-dT with the Multiscribe RT system (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The RT reaction was performed at 25˚C for 5 min, followed by incubations at 46˚C for 20 min and at 95˚C for 1 min. An iCycler and its system interface software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used to perform qPCR. Amplification was performed as follows: Uracil-DNA glycosylase activation at 50˚C for 2 min, initial denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. Using the AmpliTaq Gold polymerase (Thermo Fisher Scientific, Inc.). Non-retrotranscribed RNA samples were used as negative control. The expression levels of the target genes were normalized to GAPDH and quantified using the $2^{-\Delta\Delta Cq}$ method (17). Standard curves for PCR were constructed to identify the gene copy number in each sample. The EC marker CD31 (cat. nos. Mm00476702_m1 and Hs00169777_m1; Thermo Fisher Scientific, Inc.) was undetectable by RT-qPCR, suggesting that only medial VSMCs were isolated without contamination of other layers (data not shown).

Statistical analysis. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as the mean ± standard error. Unpaired two-tailed Student's t-test was performed to compare two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

FGF signaling induces a synthetic phenotype in VSMCs and promotes inflammation. To investigate the role of FGF in VSMCs phenotypic switching and the expression levels of inflammatory molecules, HASMCs were cultured with or without FGF1 at a concentration of 50 ng/ml. RT-qPCR and ELISA were used to examine the phenotype of cultured VSMCs. The mRNA expression level of TGFβR2, involved in VSMC differentiation (16), was decreased in cells treated with FGF compared with untreated controls. Consistently with the decreased expression level of TGFβR2, the mRNA

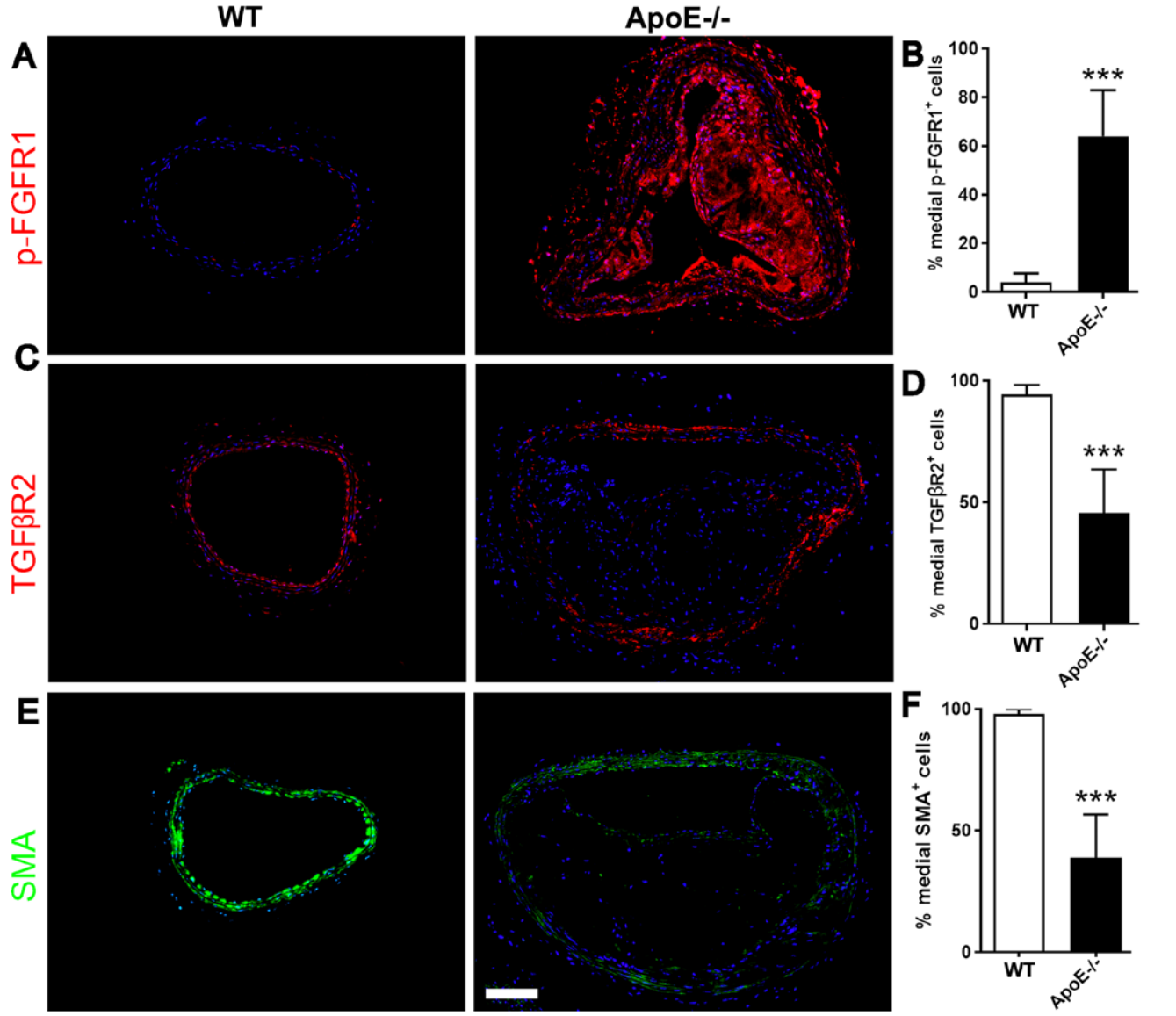

Figure 3. VSMCs in hypercholesterolemic mice exhibit activated FGFR2 and decreased expression level of contractile proteins. Immunofluorescence analysis of VSMCs in brachiocephalic arteries of WT fed a standard chow or ApoE$^{-/-}$ mice fed a high-fat diet for 4 months. (A) Representative images of p-FGFR1 staining and (B) rate of p-FGFR1-positive medial cells. (C) Representative images of TGFβR2 staining and (D) rate of TGFβR2-positive medial cells. (E) Representative images of SMA staining and (F) rate of ACTA2-positive medial cells. n=8 mice in each group. Sections were counterstained with DAPI to visualize the nuclei in blue. Scale bar, 100 μm. Data are presented as the mean ± standard error. Statistical significance was determined by unpaired two-tailed Student's t-test. ***P<0.001 vs. WT. WT, wild-type; ApoE, apolipoprotein E; p-FGFR1, phosphorylated fibroblast growth factor receptor 1; TGFβR2, transforming growth factor β receptor 2; SMA, smooth muscle actin.

expression levels of genes encoding contractile proteins, including CNN1 and ACTA2, were decreased following treatment with FGF (Fig. 1A). Treatment with FGF significantly increased the protein and mRNA expression levels of the inflammatory chemokines CCL2, CXCL9, CXCL10 and CXCL11 (Fig. 1B and C). The present data suggested that treatment with FGF induced TGFβR2 inhibition and loss of contractile markers. In addition, FGF activation induced VSMCs to express various chemokines.

Inhibition of FGF signaling in VSMCs maintains their contractile phenotype and suppresses inflammation. To further investigate the role of FGF signaling in the phenotypic transition of VSMCs and in the synthesis of inflammatory cytokines, FRS2 shRNA was used to knockdown the expression level of FRS2 in HASMCs. RT-qPCR and ELISA were performed to examine the expression levels of various chemokines in cultured VSMCs. A significant decrease in the expression level of FRS2 was detected in VSMCs transfected with FRS2 shRNA (Fig. 2A). Additionally, the expression levels of genes encoding the contractile proteins CNN1 and SMA (encoded by ACTA2) were significantly increased following FRS2 knockdown. In addition, the expression level of TGFβR2 was upregulated following FRS2 knockdown cells compared with control cells (Fig. 2B). The present results further suggested that FGF signaling may inhibit TGFβ signaling and influence the phenotype of VSMCs. Furthermore, the expression levels of genes encoding chemokines was significantly decreased following FRS2 knockdown (Fig. 2C). Additionally, the secretion levels of CXCL9, CXCL10 and CXCL11 were undetectable in the FRS2

knockdown group (Fig. 2D). The present results suggested that activation of the FGF signaling in VSMCs caused a significant decrease in the expression levels of contractile markers and an increase in inflammatory markers. Collectively, the present results suggested that FGF signaling may serve a role in the phenotypic switching of VSMCs by increasing the secretion of chemokines by VSMCs. In addition, inhibition of FGF signaling was sufficient to significantly decrease inflammation in VSMCs, indicated by the changes in chemokines.

Increased FGF signaling in aortic VSMCs of ApoE$^{-/-}$ mice. FGF signaling was previously demonstrated to be involved in atherosclerosis (15). The ApoE$^{-/-}$ mouse model is the most common murine model used to investigate atherosclerosis. To examine the role of FGF and TGFβ signaling in the phenotype of VSMCs *in vivo*, ApoE$^{-/-}$ mice and WT C57BL/6J mice were used to compare the protein expression level of p-FGFR1 and TGFβR2 in medial VSMCs. Immunofluorescence staining was performed to investigate the protein expression levels of p-FGFR1, TGFβR2 and ACTA2 in VSMCs. The medial VSMCs in ApoE$^{-/-}$ mice exhibited an increased protein expression level of p-FGFR1, whereas SMA (encoded by ACTA2) was decreased (Fig. 3A and B). The protein expression levels of TGFβR2 and SMA were decreased in VSMCs of ApoE$^{-/-}$ mice compared with WT mice (Fig. 3C-F). The present results suggested that FGF signaling was activated in the medial VSMCs of aortic arteries exhibiting atherosclerotic plaques, as assessed by SMA staining. Additionally, activation of FGF signaling inhibited the expression level of TGFβR2 and contractile proteins, suggesting the initiation of a phenotypic switch in medial VSMCs.

Aortic VSMCs express chemokines in ApoE$^{-/-}$ mice. To examine the protein expression levels of inflammatory molecules in medial VSMCs *in vivo,* ApoE$^{-/-}$ mice and WT mice were used to investigate the expression levels of various genes encoding chemokines in the medial VSMCs of aortic arteries. To detect the mRNA expression levels of genes associated with inflammation in medial VSMCs and not in surrounding cells in the intima and adventitia, laser-capture microdissection was used to collect medial VSMCs. qPCR analysis of dissected medial tissues suggested that VSMCs in WT mice exhibited significant decreased expression levels of CCL2, CXCL9, CXCL10 and CXCL11, compared with ApoE$^{-/-}$ mice (Fig. 4). In addition, VSMCs in ApoE$^{-/-}$ mice exhibited increased p-FGFR1 and decreased expression of contractile proteins. Collectively, the present data were consistent with the aforementioned *in vitro* results and demonstrated that active FGF signaling altered the phenotype of VSMCs by inducing the expression of various inflammatory molecules.

Increased FGF signaling in medial VSMCs of human coronary arteries. To further investigate the role of FGF and TGFβ signaling in the phenotype of VSMCs in human, coronary arteries were collected from patients with atherosclerotic plaques and healthy donors. Subsequently, the expression levels of p-FGFR1 and TGFβR2 in medial layer VSMCs were analyzed. Immunofluorescence staining was used to examine the protein expression levels of p-FGFR1, TGFβR2 and SMA. Consistent with the aforementioned results in mouse, the medial VSMCs

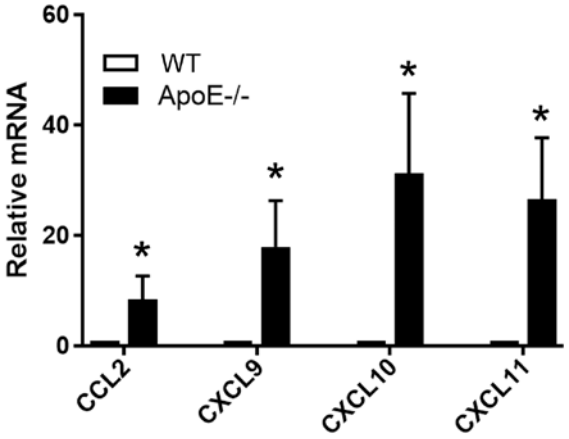

Figure 4. Vascular smooth muscle cells in hypercholesterolemic mice express chemokines. Laser-capture microdissection method was used for dissection of the medial layer of arteries collected from WT fed a standard chow or ApoE-/- mice fed a high-fat diet for 4 months. Expression levels of gene encoding chemokines were assessed by reverse transcription-quantitative PCR. n=3 mice per group. GAPDH was used as the internal control. Data are presented as fold changes in ApoE$^{-/-}$ mice relative to C57BL/6 WT mice. Data are presented as the mean ± standard error. Statistical significance was determined by unpaired two-tailed Student's t-test. *P<0.05 vs. WT. WT, wild-type; ApoE, apolipoprotein E; CCL2, C-C motif chemokine ligand 2; CXCL, C-X-C motif chemokine ligand.

in patients with atherosclerotic plaques exhibited upregulated p-FGFR1 and downregulated SMA expression (Fig. 5A and B). The protein expression levels of TGFβR2 exhibited the same trend of SMA, with decreased expression in the medial VSMCs of arteries from patients with atherosclerosis compared with healthy controls (Fig. 5C-F). The present results suggested that FGF signaling was activated in the medial VSMCs of coronary arteries of patients with advanced atherosclerotic plaques. Collectively, FGF signaling may inhibit the protein expression level of TGFβR2 and contractile factors, inducing a phenotypic switch in medial VSMCs.

Medial VSMCs release chemokines in human coronary arteries. To examine the secretion of inflammatory molecules by medial VSMCs in human, coronary arteries with advanced plaques and healthy arteries were collected, and the expression levels of various chemokines in the medial VSMCs of coronary arteries were investigated. Laser-capture microdissection was used to remove the medial layer of human coronary arteries and to collect only medial VSMCs. RT-qPCR was used to measure the mRNA expression levels of inflammatory cytokines synthesized by medial VSMCs, and not by additional cells in the intima and adventitia. The mRNA expression levels of genes encoding the chemokines CCL2, CXCL9, CXCL10 and CXCL11 were significantly increased in coronary arteries from patients with atherosclerosis compared with healthy controls (Fig. 6). The increase in the expression levels of genes encoding chemokines in human patients was observed in addition to the activation of FGF signaling and the decrease in the expression level of SMA. Collectively, the present results are consistent with the aforementioned analyses in mice and suggested that upregulation of FGF signaling may induce VSMCs phenotypic switching by promoting the secretion of inflammatory molecules by VSMCs.

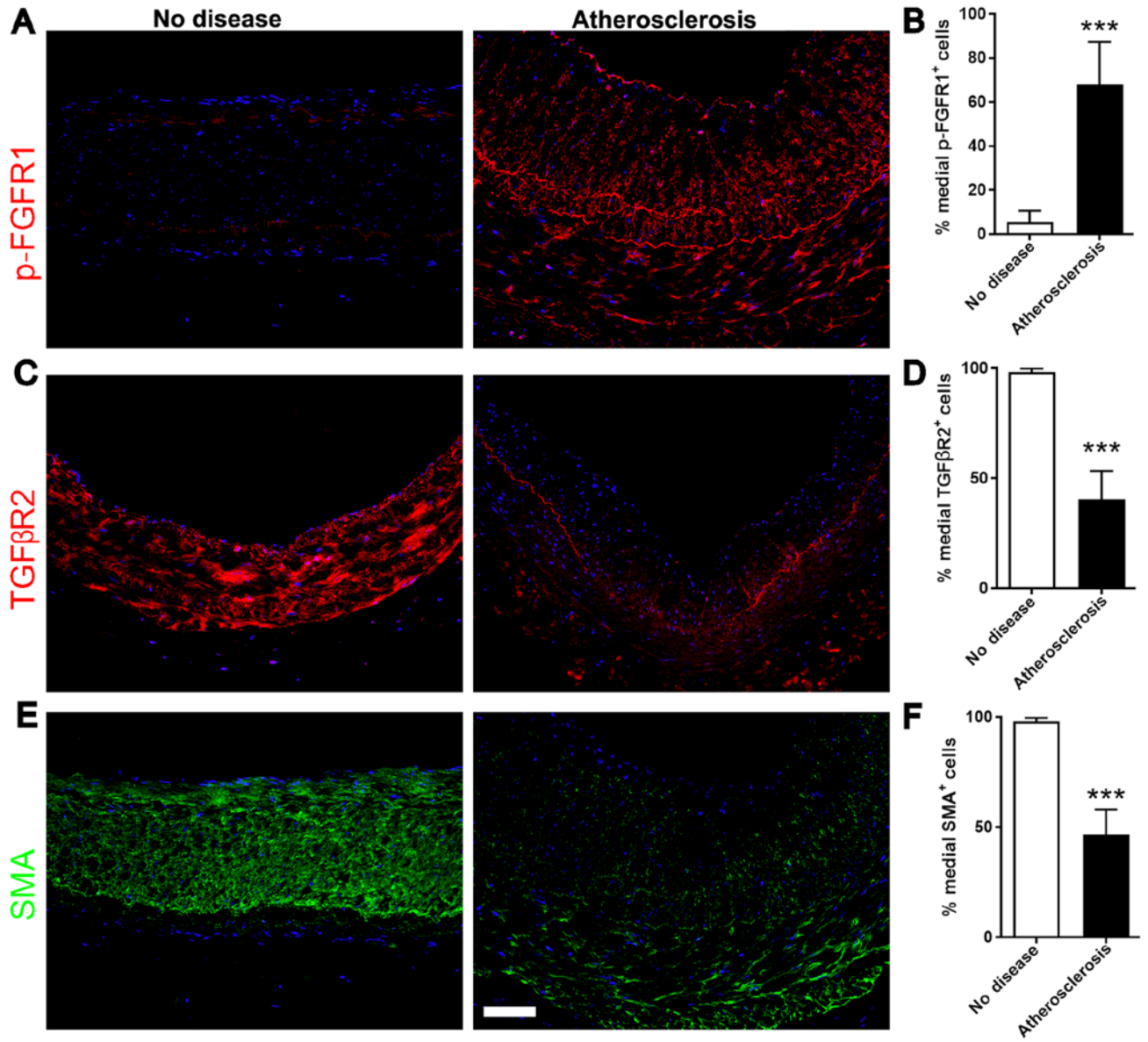

Figure 5. VSMCs in human coronary arteries exhibit activated FGF receptors and decreased expression levels of contractile proteins. Immunofluorescence analysis on VSMCs in human coronary arteries with or without atherosclerosis. (A) Representative images of p-FGFR1 staining and (B) rate of p-FGFR1-positive medial cells. (C) Representative images of TGFβR2 staining and (D) rate of TGFβR2-positive medial cells. (E) Representative images of SMA staining and (F) rate of ACTA2-positive medial cells. n=6 donors in each group. Sections were counterstained with DAPI to visualize the nuclei in blue. Scale bar, 100 μm. Data are presented as the mean ± standard error of the mean. Statistical significance was determined by unpaired two-tailed Student's t-test. ***P<0.001 vs. healthy controls. p-FGFR1, phosphorylated fibroblast growth factor receptor 1; TGFβR2, transforming growth factor β receptor 2; SMA, smooth muscle actin.

Discussion

The role of the phenotypic switching of VSMCs in the process of atherosclerosis is not fully understood. In the present study, FGF signaling was identified to induce phenotypic switching of cultured VSMCs from a contractile state to a secretory state, characterized by chemokine production. Suppressing FGF signaling in VSMCs decreased the secretion of chemokines. Furthermore, analysis of arteries from ApoE$^{-/-}$ mice and human atherosclerotic patients suggested that medial VSMCs exhibited an increase in the activation of FGF signaling and in the expression levels of pro-inflammatory factors. Collectively, the present results suggested that FGF signaling may have a significant role in the regulation of VSMCs dedifferentiation and in the expression of pro-inflammatory factors. Additionally, the present results suggested that FGF signaling may be a pro-atherogenic factor, able to suppress contractile markers and to induce a secretory phenotype in VSMCs.

The FGF family includes 28 multifunctional ligands that function as growth factors (18). FGF receptors are a family of receptor tyrosine kinases consisting of five members (19-22). In the arterial wall, endothelial cells and VSMCs express FGFs and FGFRs (23). The FGFRs expressed at high levels in VSMCs are FGFR1, FGFR2 and FGFR3, which are activated by FGF1 (14). FGF signaling was previously demonstrated to induce dedifferentiation VSMCs from a contractile phenotype to a proliferative and synthetic state by inhibiting TGFβ signaling (14,15). In the present study, activated FGF signaling was identified to increase the secretion of various pro-inflammatory factors. The present results suggested that

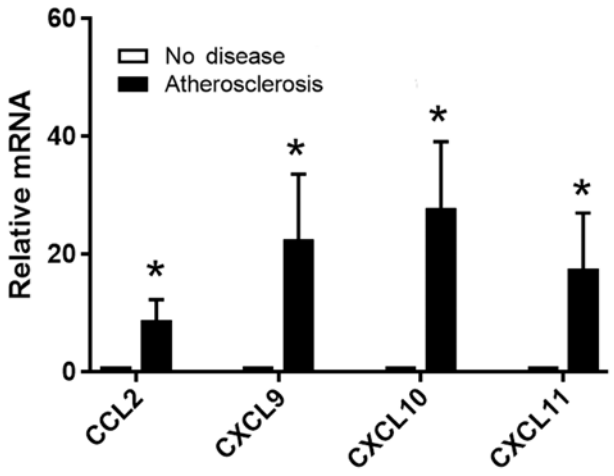

Figure 6. Vascular smooth muscle cells in human coronary arteries release chemokines. Laser-capture microdissection methods were used for dissection of the medial layer of human coronary arteries collected from health subjects or from patients with atherosclerosis. Expression levels of genes encoding chemokines were assessed by reverse transcription-quantitative polymerase chain reaction. n=3 donors in each group. GAPDH was used as the internal control. Data are presented as fold changes in patients with atherosclerosis relative to healthy controls. Data are presented as the mean ± standard error of the mean. Statistical significance was determined by unpaired two-tailed Student's t-test. *P<0.05 vs. no disease (healthy controls). CCL2, C-C motif chemokine ligand 2; CXCL, C-X-C motif chemokine ligand.

FGF signaling may induce an increase in the protein expression levels of multiple chemokines in VSMCs, promoting leukocyte trafficking to inflammatory sites, including atherosclerotic plaques.

VSMCs are the only cell type in the medial layer of arteries. Arterial VSMCs exhibit contractile functions and certain synthetic functions, including elastin and collagen deposition. Under homeostatic conditions, VSMCs maintain a quiescent phenotype and are involved in extracellular matrix deposition and in the turnover of collagen and elastin (7). Various stresses, including inflammatory stimuli from microbial infection and infiltrated leukocytes, are able to induce a pro-inflammatory response in VSMCs, initiating phenotype switching, and promoting the release of numerous cytokines and chemokines (6,24,25). The expression levels of inflammatory factors expressed by endothelial cells and synthetic VSMCs are similar (26,27). Similarly to endothelial cells, VSMCs can secrete large amounts of pro-inflammatory factors, recruiting macrophages and T cells to the vessel wall, particularly to the neointima and adventitia (28-30).

Under atherosclerotic conditions, VSMCs in the medial layer of arteries undergo a phenotypic switch in response to various stimuli, including hypercholesterolemia, activated endothelial cells and infiltrated leukocytes (3). In the present study, synthetic VSMCs were identified to secrete increased levels of pro-inflammatory molecules compared with contractile VSMCs, and synthetic VSMCs may serve a significant role in initiating atherosclerosis. Various chemokines expressed by VSMCs may initiate and promote atherosclerosis. CCL2 is a potent chemoattractant that is able to recruit monocytes and macrophages to the vessel wall, and induce them to infiltrate into the neointima through the arterial lumen and into the adventitia through the postcapillary venules (31-34). Macrophages transdifferentiate into foam cells in microenvironments exhibiting high circulating levels of cholesterol and may promote plaque development. CXCL9, CXCL10 and CXCL11 are important chemoattractants that recruit CXC receptor type 3-expressing T cells into the vessel wall (34-38). These T cells may induce the release of interferon γ (IFNγ) and tumor necrosis factor α (TNFα) derived from macrophages and VSMCs, promoting the progression of atherosclerotic plaques (5-7). Notably, the level of CXCL10 secreted by synthetic VSMCs is between 10- and 100-fold higher compared with macrophages and T cells in atherosclerotic arteries, whereas macrophages and T cells are the principal cell types that secrete IFNγ and TNFα (7). This crosstalk between leukocytes and VSMCs may induce a positive feedback loop in arteries causing progression of atherosclerotic plaques.

Collectively, the findings of the present study suggested that FGF signaling-induced phenotype switching leads to increased secretion of chemokines by VSMCs, which may be involved in leukocyte infiltration into the vascular lumen and initiation of atherosclerotic lesion development. The present results suggested that FGF signaling may promote atherosclerosis by increasing VSMC proliferation and by inducing chemokine expression in VSMCs. Additionally, the present study suggested that modulation of the FGF signaling pathway may be an effective strategy for the treatment and prevention of atherosclerosis.

Acknowledgements

Not applicable.

Funding

The present study was supported by The National Natural Science Foundation of China (grant no. 81770488) and the Liaoning Province Clinical Training Project (grant no. LNCCC-C03-2015).

Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

MQ and SX designed the study and prepared the manuscript. MQ performed the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The Ethics Committee of The First Hospital of China Medical University (Shenyang, China). All patients or family members of the organ donors provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Libby P, Ridker PM and Hansson GK: Progress and challenges in translating the biology of atherosclerosis. Nature 473: 317-325, 2011.
2. Sanz J and Fayad ZA: Imaging of atherosclerotic cardiovascular disease. Nature 451: 953-957, 2008.
3. Bennett MR, Sinha S and Owens GK: Vascular smooth muscle cells in atherosclerosis. Circ Res 118: 692-702, 2016.
4. Liu R, Leslie KL and Martin KA: Epigenetic regulation of smooth muscle cell plasticity. Biochim Biophys Acta 1849: 448-453, 2015.
5. Burns WR, Wang Y, Tang PC, Ranjbaran H, Iakimov A, Kim J, Cuffy M, Bai Y, Pober JS and Tellides G: Recruitment of CXCR3+ and CCR5+ T cells and production of interferon-gamma-inducible chemokines in rejecting human arteries. Am J Transplant 5: 1226-1236, 2005.
6. Ahmad U, Ali R, Lebastchi AH, Qin L, Lo SF, Yakimov AO, Khan SF, Choy JC, Geirsson A, Pober JS and Tellides G: IFN-gamma primes intact human coronary arteries and cultured coronary smooth muscle cells to double-stranded RNA- and self-RNA-induced inflammatory responses by upregulating TLR3 and melanoma differentiation-associated gene 5. J Immunol 185: 1283-1294, 2010.
7. Tellides G and Pober JS: Inflammatory and immune responses in the arterial media. Circ Res 116: 312-322, 2015.
8. Kawai-Kowase K and Owens GK: Multiple repressor pathways contribute to phenotypic switching of vascular smooth muscle cells. Am J Physiol Cell Physiol 292: C59-C69, 2007.
9. Lindner V and Reidy MA: Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. Proc Natl Acad Sci USA 88: 3739-3743, 1991.
10. Jackson CL and Reidy MA: Basic fibroblast growth factor: Its role in the control of smooth muscle cell migration. Am J Pathol 143: 1024-1031, 1993.
11. Chen PY, Qin L, Barnes C, Charisse K, Yi T, Zhang X, Ali R, Medina PP, Yu J, Slack FJ, *et al*: FGF regulates TGF-β signaling and endothelial-to-mesenchymal transition via control of let-7 miRNA expression. Cell Rep 2: 1684-1696, 2012.
12. Chen PY, Qin L, Tellides G and Simons M: Fibroblast growth factor receptor 1 is a key inhibitor of TGFβ signaling in the endothelium. Sci Signal 7: ra90, 2014.
13. Chen PY, Qin L, Baeyens N, Li G, Afolabi T, Budatha M, Tellides G, Schwartz MA and Simons M: Endothelial-to-mesenchymal transition drives atherosclerosis progression. J Clin Invest 125: 4514-4528, 2015.
14. Chen PY, Qin L, Li G, Tellides G and Simons M: Fibroblast growth factor (FGF) signaling regulates transforming growth factor beta (TGFβ)-dependent smooth muscle cell phenotype modulation. Sci Rep 6: 33407, 2016.
15. Chen PY, Qin L, Li G, Tellides G and Simons M: Smooth muscle FGF/TGFβ cross talk regulates atherosclerosis progression. EMBO Mol Med 8: 712-728, 2016.
16. Lebastchi AH, Khan SF, Qin L, Li W, Zhou J, Hibino N, Yi T, Rao DA, Pober JS and Tellides G: Transforming growth factor beta expression by human vascular cells inhibits interferon gamma production and arterial media injury by alloreactive memory T cells. Am J Transplant 11: 2332-2341, 2011.
17. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
18. Ornitz DM and Itoh N: The fibroblast growth factor signaling pathway. Wiley Interdiscip Rev Dev Biol 4: 215-266, 2015.
19. Chen PY, Simons M and Friesel R: FRS2 via fibroblast growth factor receptor 1 is required for platelet-derived growth factor receptor beta-mediated regulation of vascular smooth muscle marker gene expression. J Biol Chem 284: 15980-15992, 2009.
20. Kouhara H, Hadari YR, Spivak-Kroizman T, Schilling J, Bar-Sagi D, Lax I and Schlessinger J: A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. Cell 89: 693-702, 1997.
21. Eswarakumar VP, Lax I and Schlessinger J: Cellular signaling by fibroblast growth factor receptors. Cytokine Growth Factor Rev 16: 139-149, 2005.
22. Gotoh N: Regulation of growth factor signaling by FRS2 family docking/scaffold adaptor proteins. Cancer Sci 99: 1319-1325, 2008.
23. Hughes SE: Localisation and differential expression of the fibroblast growth factor receptor (FGFR) multigene family in normal and atherosclerotic human arteries. Cardiovasc Res 32: 557-569, 1996.
24. Zhou J, Tang PC, Qin L, Gayed PM, Li W, Skokos EA, Kyriakides TR, Pober JS and Tellides G: CXCR3-dependent accumulation and activation of perivascular macrophages is necessary for homeostatic arterial remodeling to hemodynamic stresses. J Exp Med 207: 1951-1966, 2010.
25. Yu L, Qin L, Zhang H, He Y, Chen H, Pober JS, Tellides G and Min W: AIP1 prevents graft arteriosclerosis by inhibiting interferon-γ-dependent smooth muscle cell proliferation and intimal expansion. Circ Res 109: 418-427, 2011.
26. Rao RM, Yang L, Garcia-Cardena G and Luscinskas FW: Endothelial-dependent mechanisms of leukocyte recruitment to the vascular wall. Circ Res 101: 234-247, 2007.
27. Qin L, Huang Q, Zhang H, Liu R, Tellides G, Min W and Yu L: SOCS1 prevents graft arteriosclerosis by preserving endothelial cell function. J Am Coll Cardiol 63: 21-29, 2014.
28. Charo IF and Taubman MB: Chemokines in the pathogenesis of vascular disease. Circ Res 95: 858-866, 2004.
29. Tabas I and Glass CK: Anti-inflammatory therapy in chronic disease: Challenges and opportunities. Science 339: 166-172, 2013.
30. He C, Medley SC, Hu T, Hinsdale ME, Lupu F, Virmani R and Olson LE: PDGFRβ signalling regulates local inflammation and synergizes with hypercholesterolaemia to promote atherosclerosis. Nat Commun 6: 7770, 2015.
31. Boring L, Gosling J, Cleary M and Charo IF: Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. Nature 394: 894-897, 1998.
32. Gerszten RE, Garcia-Zepeda EA, Lim YC, Yoshida M, Ding HA, Gimbrone MA Jr, Luster AD, Luscinskas FW and Rosenzweig A: MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. Nature 398: 718-723, 1999.
33. Zheng Y, Qin L, Zacarías NV, de Vries H, Han GW, Gustavsson M, Dabros M, Zhao C, Cherney RJ, Carter P, *et al*: Structure of CC chemokine receptor 2 with orthosteric and allosteric antagonists. Nature 540: 458-461, 2016.
34. Veillard NR, Steffens S, Pelli G, Lu B, Kwak BR, Gerard C, Charo IF and Mach F: Differential influence of chemokine receptors CCR2 and CXCR3 in development of atherosclerosis in vivo. Circulation 112: 870-878, 2005.
35. Heller EA, Liu E, Tager AM, Yuan Q, Lin AY, Ahluwalia N, Jones K, Koehn SL, Lok VM, Aikawa E, *et al*: Chemokine CXCL10 promotes atherogenesis by modulating the local balance of effector and regulatory T cells. Circulation 113: 2301-2312, 2006.
36. Zernecke A, Bot I, Djalali-Talab Y, Shagdarsuren E, Bidzhekov K, Meiler S, Krohn R, Schober A, Sperandio M, Soehnlein O, *et al*: Protective role of CXC receptor 4/CXC ligand 12 unveils the importance of neutrophils in atherosclerosis. Circ Res 102: 209-217, 2008.
37. Schwarz JB, Langwieser N, Langwieser NN, Bek MJ, Seidl S, Eckstein HH, Lu B, Schömig A, Pavenstädt H and Zohlnhöfer D: Novel role of the CXC chemokine receptor 3 in inflammatory response to arterial injury: Involvement of mTORC1. Circ Res 104: 189-200, 2009.
38. Tavakolian Ferdousie V, Mohammadi M, Hassanshahi G, Khorramdelazad H, Khanamani Falahati-Pour S, Mirzaei M, Allah Tavakoli M, Kamiab Z, Ahmadi Z, Vazirinejad R, *et al*: Serum CXCL10 and CXCL12 chemokine levels are associated with the severity of coronary artery disease and coronary artery occlusion. Int J Cardiol 233: 23-28, 2017.