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Cyclic stretch upregulates SDF-1α/CXCR4 axis in human saphenous vein smooth muscle cells

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ABSTRACT

Cyclic stretch (CS) mediates different cellular functions in vascular smooth muscle cells and involves in neointimal hyperplasia and subsequent atherosclerosis of vein grafts. Here, we investigated whether CS can modulate stromal cell-derived factor-1α (SDF-1α)/CXCR4 axis in human saphenous vein smooth muscle cells. We found CS induced the upregulation of SDF-1α and CXCR4 in human saphenous vein smooth muscle cells *in vitro*, which was dependent on PI3K/Akt/mTOR pathway. Furthermore, CS augmented human saphenous vein smooth muscle migration and focal adhesion kinase (FAK) activation by PI3K/Akt/mTOR pathway. Interestingly, the upregulation of SDF-1α/CXCR4 axis was instrumental in CS-induced saphenous vein smooth muscle cell migration and FAK activation, as showed by AMD3100, an inhibitor of SDF-1α/CXCR4 axis, partially but significantly blocked the CS-induced cellular effects. Thus, those data suggested SDF-1α/CXCR4 axis involves in CS-mediated cellular functions in human saphenous vein smooth muscle cells.

At present, autologous saphenous veins remain the most widely used bypass conduit for the treatment of coronary artery disease [1]. However, occlusions in such grafts are common, resulting in patency rates of only 50% after 10 years [2]. Usually vein graft occlusion occurs as a result of intimal hyperplasia and subsequent atherosclerosis, which is characterized by the migration and proliferation of vascular smooth muscle cells (SMCs) [3,4]. After implantation, vein grafts undergo a sharp increase in mechanical tension owing to higher flow velocities in the arterialized circulation. These hemodynamic alterations may be, at least in part, responsible for short and long-term functional and morphological vein graft remodeling that cause intimal hyperplasia and atherogenesis [5]. When implanted in the arterial circulation, vein SMCs are exposed to two kinds of abrupt increase of mechanical forces, cyclic stretch from the circumferential force and hydrostatic pressure from the perpendicular force. In vascular SMCs, different cellular functions have been shown to be modulated by cyclic stretch, which include proliferation, apoptosis, migration, etc. [6]. Although a paucity of evidence has shown that CS can promote migration of SMCs [6], the mechanism remains poorly understood.

Stromal cell-derived factor-1α (SDF-1α) and its receptor CXCR4, which are essential for stem cell mobilization/homing and organ system vascularization [7,8], are also highly expressed in injured arteries and atherosclerotic plaques [9]. After arterial injury highly expressed SDF-1α can recruit a subset of SMC progenitors, which plays an instrumental role in neointimal hyperplasia [10,11]. Recently, Napolitano et al. found the athero-protective effect of laminar shear stress was dependent on down-regulation of CXCR4, which suggested SDF-1α/CXCR4 axis could mediate atherosclerotic progress and be influenced by some kinds of mechanical forces [12].

Focal adhesion kinase (FAK) is a critical mediator of signaling events between cells and their extracellular matrix [13]. The phosphorylation and activation of FAK play an important role in vascular SMCs migration and intimal hyperplasia [14]. It has been reported in lymphocytes and some types of cancer cells, SDF-1α could activate FAK and paxillin, promoting the cellular motility [15,16].

Given the outlined above, we hypothesized cyclic stretch might exert some of its biological effects by affecting SDF-1α/CXCR4 axis in SMCs. Here, we found cyclic stretch upregulated SDF-1α and CXCR4 expression in human saphenous vein SMCs via PI3K/Akt/mTOR pathway; the SDF-1α/CXCR4 axis mediated CS-induced migration and activation of FAK in human saphenous SMCs.

Materials and methods

Cell culture. Redundant human saphenous veins were obtained from patients undergoing coronary bypass surgery in accordance with the guidelines of the local Ethics Committee. Human

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saphenous vein SMCs were grown from these segments by the explant method as previously described [17]. Human saphenous vein SMCs were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 100 μg/mL of penicillin, 100 IU/mL streptomycin, 2 mmol/L L-glutamine, and 10% (vol/vol) fetal calf serum (FCS) (Gibco, USA). SMCs were used at passage 4–8.

Cell culture under cyclic stretch. Human saphenous vein SMCs cultured on BioFlex collagen type I 6-well plates (Flexcell, Hillsborough, NC) (density, 4×10^4/mL). After cell confluence human saphenous vein SMCs were subjected to serum-starvation for 12 h; serum-free culture medium was changed and stretching was performed with a FX-3000 FlexerCell strain unit with 15% cyclic elongation at 0.5 Hz, which corresponds to the physiological maximum deformation in the human coronary artery. Control BioFlex plates were in static condition, namely, the cells were treated similarly just without CS.

Preparation of protein extracts and Western blotting. For detecting the expression of CXCR4, Akt, phosphorylation Akt, total mTOR, phosphor-mTOR (Ser 2448), total p70S6K, phosphor-p70S6K (Thr 389), total FAK, and phospho-FAK (Tyr 397). SDS–PAGE was performed with 50 μg of protein extracted from human saphenous vein SMCs. After pretreatment the cells were washed extensively with ice-cold phosphate-buffered saline (PBS) and lysed on ice with 300 μl of 30 mM MOPS, pH 7.0, 0.15 M NaCl, 1 mM Na_3VO_4, 5 mM NaF, 1 mM EDTA, 1% NP 40, and protease inhibitors. Blots were incubated with anti-CXCR4 c-20 (Santa Cruz Biotechnology); Akt and phosphorylation Akt (Ser 473) antibodies, mTOR, phosphor-mTOR (Ser 2448) antibodies (Santa Cruz Biotechnology), p70S6K, phosphor-p70S6K antibodies (Thr 389) (Santa Cruz Biotechnology); FAK (BD Pharmingen) and phospho-FAK (Tyr 397) (Biosource) antibodies.

Real-time polymerase chain reaction. Total RNA from human saphenous vein SMCs was prepared utilizing the Trizol (Invitrogen) method, followed by genomic DNA digestion using DNAse I (Invitrogen, Karlsruhe, Germany). Total RNA was determined by the OD260 nm method. cDNA synthesis was performed using 1 μg of RNA in a total volume of 10 μl with MMLV RT (Invitrogen). Primer concentration for Q-PCR was 15 pM/20 μl. cDNA (2 μL) from RT reactions was used in reactions conducted with the Roche Lightcycler; the procedure was denaturation 5 min at 94 °C followed by 40 cycles of 5 s at 94 °C, 10 s at 58 °C, and 15 s at 72 °C. For each assayed gene, annealing temperature and the number of cycles resulting in linear amplification were tested. The primers were GAPDH: 5′-GTCGGTGTGAACGGATTTG-3′, 5′-CTTGCCGTGGGTAGAGTCAT-3′; SDF-1α: 5′-GCTCTGCATCAGTGACGGTAAG-3′; 5′-TGGCGACATGGCTCTCAAA-3′. Results were normalized to GAPDH within the same sample.

FAK activity. FAK was immunoprecipitated from 50 μg protein samples as indicated earlier. Immunoprecipitates were incubated with paxillin in kinase buffer (50 mmol/L MOPS, pH 7.5, 10 mmol/L $MnCl_2$, 10 mmol/L $MgCl_2$) containing [γ^{32}P]ATP for 30 min at 30 °C. Kinase reactions were stopped by addition of 2× Laemmli buffer, and samples were separated by SDS–PAGE electrophoresis and visualized by autoradiography. After autoradiography, gel proteins were transferred onto a polyvinylidene difluoride membrane and probed with anti-FAK to confirm equal loading.

Human saphenous vein SMCs migration assay. After human saphenous vein SMCs were exposed to CS or static conditions for different time, the cells were stripped with Cellstripper™ (Fisher Scientific, Pittsburgh, PA, USA), a nonenzymatic cell dissociation solution that gently dislodges adherent cells in tissue culture. Then, cells were added to the upper well of Transwells (12-mm diameter, 8.0-μm pore size, Costar Corning, Cambridge, MA) at 1×10^5 cells per well.

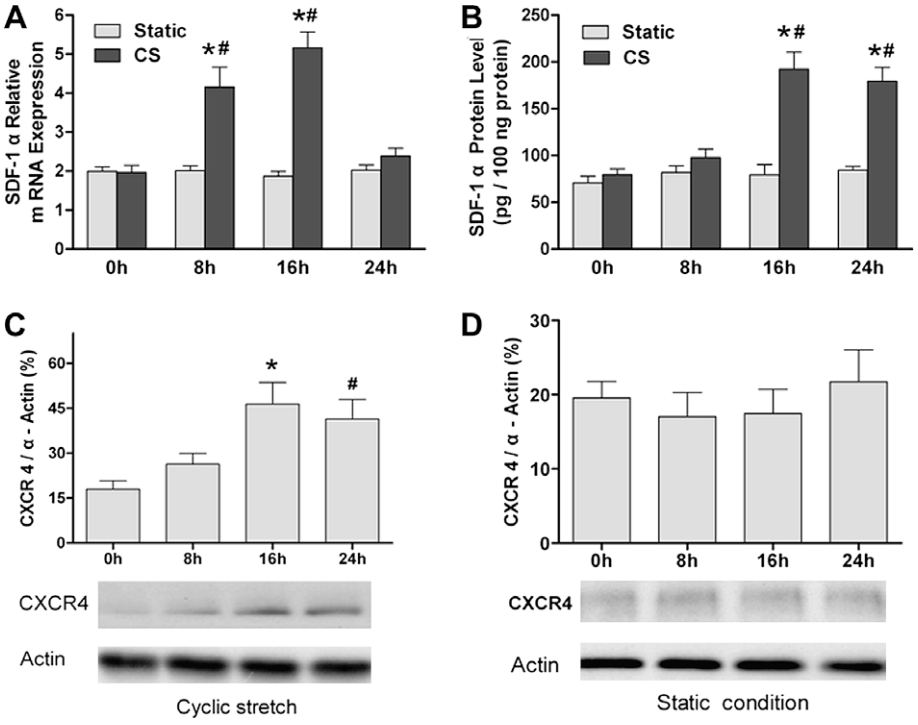

Fig. 1. CS induced upregulation of SDF-1α/CXCR4 axis. (A) Confluent human saphenous vein SMCs were subjected to CS for 0 h, 8 h, 16 h and 24 h. Whole cell lysates were collected to measure SDF-1α mRNA levels by real-time PCR ($n = 8$, $^*P < 0.05$ vs group 0 h, $^\#P < 0.05$ vs static group). (B) The time course of SDF-1α protein secretion induced by CS. The supernatants were collected to evaluate secretion of SDF-1α by ELISA after confluent human saphenous vein SMCs exposing to CS for 0 h, 8 h, 16 h and 24 h ($n = 8$, $^*P < 0.05$ vs group 0 h, $^*P < 0.05$ vs static group). (C) CS induced the expression of CXCR4 in human saphenous vein SMCs in a time-dependent manner. The expression of CXCR4 was analyzed by Western blot analysis ($n = 4$, $^{*,\#}P < 0.05$ vs group 0 h). (D) There was no significant difference in CXCR4 expression under static condition at different time points.

Some samples were treated with AMD3100. The Transwell was incubated for 36 h at 37 °C in a CO_2 incubator. At the end of the experiment, the filters were fixed and stained. Cells migrating to the lower chamber were counted using a Nikon TE300 microscope at magnification 10×. Experiments were done in six wells. Ten random fields were read in each well. The average number of cells per well was determined by multiplying the average number of cells per 10× field by the number of fields per well.

Statistical analysis. Data are expressed as means ± SE. The number of samples examined is indicated by *n*. Data were analyzed by ANOVA, followed by a modified Student's *t*-test. A probability value of $P < 0.05$ was considered statistically significant.

Results

CS upregulated SDF-1α/CXCR4 axis

To determine whether CS could induce the activation of SDF-1α/CXCR4 axis in human saphenous vein SMCs, the expression of SDF-1α and CXCR4 were evaluated. we found CS significantly pro-

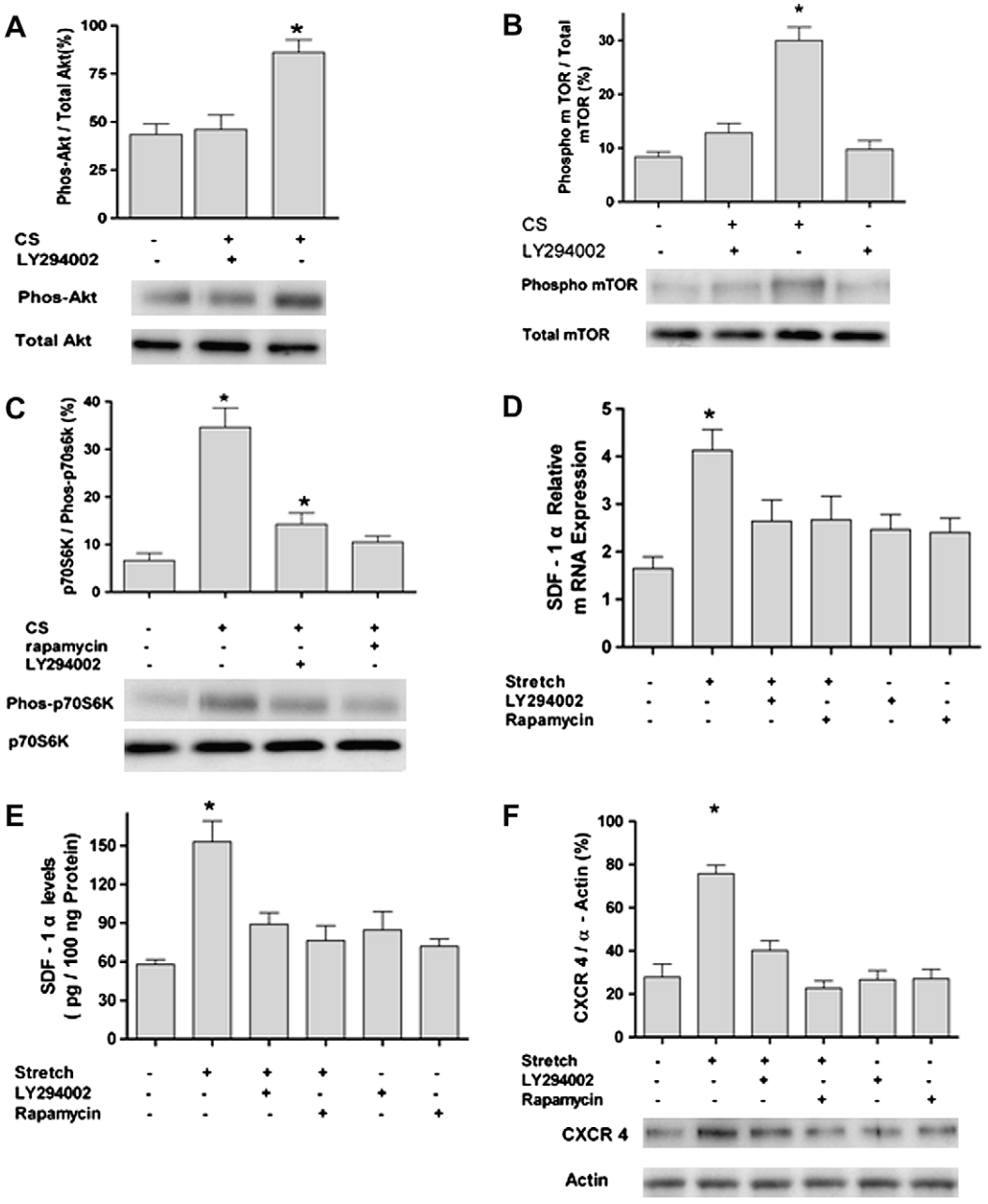

Fig. 2. The upregulation of SDF-1α/CXCR4 axis was dependent on PI3K/Akt/mTOR signaling pathway. Confluent human saphenous vein smooth muscle cells (SMCs) were exposed to static condition or CS for 30 min; total Akt, phospho-Akt (Ser 473), total mTOR, phosphor-mTOR (Ser 2448), total p70S6K and phosphor-p70S6K (Thr 389) were analyzed by Western blot. Nextly, confluent human saphenous vein SMCs were pretreated by rapamycin (10 nM) or LY294002 (10 μM) for 1 h and then subjected to CS. Cells without exposing to CS and treatment with rapamycin and LY294002 were used as control group. (A) Thirty minutes treatment with CS induced phosphorylation of Akt at Ser 473 in human saphenous vein SMCs ($n = 4$, $^*P < 0.05$). (B) CS stimulated phosphorylation of mTOR at Ser 2448, while LY294002 abolished the effects of CS ($n = 4$, $^*P < 0.05$). (C) The phosphorylation of p70S6K at Thr 389 induced by CS was blocked by LY294002 (10 μM) and rapamycin (10 nM) ($n = 4$, $^*P < 0.05$). (D) Real-time PCR was used to measure the expression of SDF-1α mRNA after SMCs exposing to CS for 12 h ($n = 8$, $^*P < 0.05$ vs control group). (E) After SMCs exposing to CS for 16 h, the secretion of SDF-1α protein was evaluated by ELISA ($n = 8$, $^*P < 0.05$ vs control group). (F) The expression of CXCR4 was measured by Western blot after SMCs exposing to CS for 16 h ($n = 4$, $^*P < 0.05$ vs control group).

moted the SDF-1α mRNA level and protein secretion, which reached peaks 12 h and 16 h after exposing to CS respectively ($n = 8$, $P < 0.05$) (Fig. 1A and B); concomitantly the expression of CXCR4 was also upregulated which got a peak 16 h after exposing to CS ($n = 4$, $P < 0.05$) (Fig. 1C). In contrast, no significant changes of SDF-1α and CXCR4 levels were observed in cells of static condition (Fig. 1A, B, and D).

The upregulation of SDF-1α/CXCR4 axis induced by CS was dependent on Akt/mTOR signaling pathway

To investigate the mechanism of the CS inducing upregulation of SDF-1α/CXCR4 axis, we firstly evaluated whether CS could activate the Akt/mTOR/p70S6K pathway. The phosphorylation of Akt, mTOR and p70S6K was augmented significantly after exposing to CS for 30 min ($n = 4$, $P < 0.05$) (Fig. 2A–C). LY294002 abolished the phosphorylation of mTOR and p70S6K (Fig. 2B and C). Nextly, we examined whether the CS-induced upregulation of SDF-1α/CXCR4 axis was through Akt/mTOR signaling pathway. LY294002 and rapamycin abolished the increase of SDF-1α mRNA and protein levels; concomitantly, the augment of CXCR4 expression was also blocked by LY294002 and rapamycin (Fig. 2D–F).

CS induced human saphenous vein SMCs migration and activation of FAK dependent on SDF-1α

At last to elucidate the role of SDF-1α/CXCR4 axis in CS-induced biological effects in human saphenous vein SMCs, we investigated the influence of CS on cell migration and FAK activation. CS promoted human saphenous vein SMCs migration with a time-dependent manner. The increase of SMCs migration got a peak at 24 h after SMCs exposing to CS (Fig. 3A). Furthermore, LY294002 and rapamycin almost totally abolished the CS-induced SMCs migration; interestingly, AMD3100, an inhibitor of SDF-1α/CXCR4 axis, partially inhibited SMCs migration induced by CS as showing by a significant difference among static condition group, CS group and CS + AMD3100 group (Fig. 3B). To investigate the effects of CS on FAK activation, we evaluated the phosphorylation and the kinase activity of FAK in human saphenous vein SMCs under CS stimulation. CS stimulated the phosphorylation of FAK and promoted FAK kinase activity, which was measured by phosphorylation of paxillin. LY294002 and rapamycin almost totally blocked the effects of CS, while AMD3100 partially but significantly attenuated the effects (Fig. 3C and D).

Discussion

In the present study, we provided the first evidence that cyclic stretch upregulated the expression of SDF-1α and CXCR4 via PI3K/Akt/mTOR pathway in human saphenous vein SMCs. We also demonstrated that CS-enhanced SMC migration and FAK activation was mediated, at least in part by upregulation of SDF-1α/CXCR4 axis in human saphenous vein SMCs.

Although a paucity of evidence has shown that CS can promote migration of arterial SMCs [18,19], the effect of mechanical stretch on venous SMC migration remains undetermined. Compared with those from arteries, SMCs from veins are exposed to a different hemodynamic environment and have different embryonic origins. Those characters determine different responses to

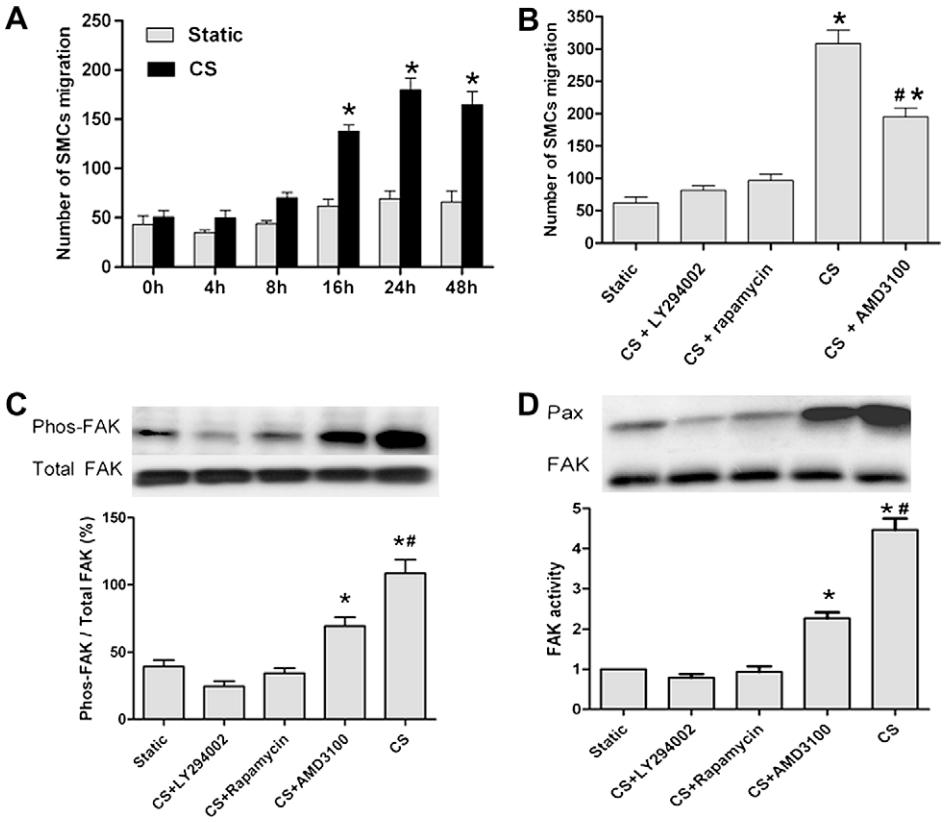

Fig. 3. CS activated FAK and promoted human saphenous vein SMCs migration. (A) The time course of CS-induced human saphenous vein SMCs migration. (B) Human saphenous vein SMCs migration was significantly augmented by CS compared to static condition ($n = 6$, $^{*}P < 0.05$ vs static group; $^{\#}P < 0.05$ vs CS + AMD3100 group). (C) Total FAK was immunoprecipitated and probed for trysine phosphorylation. FAK phosphorylation induced by CS was inhibited by AMD3100 (50 μmmol/L) ($n = 4$, $^{*}P < 0.05$ vs static group; $^{\#}P < 0.05$ vs CS + AMD3100 group). (D) Activity of immunoprecipitated FAK was detected by kinase assay with paxillin as substrate ($n = 4$, $^{*}P < 0.05$ vs static group; $^{\#}P < 0.05$ vs CS + AMD3100 group).

stimuli between arterial and venous SMCs. It has been reported in response to stimulation, compared with arterial SMCs, venous SMCs appeared to modulate gene expression and functional responses in directions more favoring atherogenesis [20]. Here, we found cyclic stretch significantly enhanced saphenous vein SMCs migration and FAK activation, which may be part of the molecular basis of neointimal hyperplasia and accelerated atherosclerosis in vein graft disease.

The most striking finding in the present study is SDF-1α/CXCR4 axis mediated CS-induced SMC migration and FAK activation. To our best known this is the first time to demonstrate that SDF-1α/CXCR4 axis served as an effector to mechanical forces. It was reported that PI3K/Akt/mTOR/p70S6K pathway could transverse the mechanical forces into cytoplasm in skeletal muscle cells [21]. Here, we demonstrated CS-induced upregulation of SDF-1α/CXCR4 axis was also dependent on PI3K/Akt/mTOR pathway in human saphenous vein SMCs, which was consistent with the previous study in spite of different type of cells. The mechanism of SDF-1α/CXCR4 axis-mediated SMCs migration and FAK activation is unclear. One possibility is SDF-1α/CXCR4 axis can activate matrix metalloproteinase family. A previous study has established SDF-1 induced a concentration and time dependent increase in proMMP-2 mRNA and protein levels in both human and murine arterial SMCs [22]. Robust evidence has showed upregulation of MMP-2 play a crucial role in SMCs migration and neointimal hyperplasia [23,24]. FAK is a ubiquitously expressed nonreceptor protein tyrosine kinase, whose function has been well studied in smooth muscle cells, where it plays an important regulatory function in cell-matrix-dependent adhesion and migration [13,14]. In cardiac myocytes and vascular endothelial cells, FAK is a major target in the mechanotransduction cascade. Although previous studies have demonstrated in lymphocytes and some types of cancer cells, SDF-1α could activate FAK and paxillin, promoting the cellular motility [15,16], to our best known, it is the first time to show that SDF-1α/CXCR4 axis mediated CS-induced FAK activation. However, except for SDF-1α/CXCR4 axis another mechanism may exist, since AMD3100 only partially blocked the CS-induced effects.

Recently several independent study groups from different points have established SDF-1α could trigger CXCR4-dependent arrest of progenitor cells on injured arteries or matrix-adherent platelets, preferentially mobilizes and recruits smooth muscle progenitors from bone marrow for neointimal SMCs [10,11]. Interestingly a recent study demonstrated bone marrow-derived cells contributed to venous intimal hyperplasia and differentiated into smooth muscle cells within the intimal lesion [25]. Those data together with our finding lead us to propose the hypothesis that the recruitment of smooth muscle progenitor cells may involve in CS-induced neointimal formation in vein grafts by upregulation SDF-1α/CXCR4 axis.

In summary, the present study demonstrated SDF-1α/CXCR4 axis was instrumental in CS-induced human saphenous vein SMCs migration and FAK activation *in vitro*. Although the precise mechanism remains unclear and further investigations *in vivo* are needed to be performed, our study firstly suggested SDF-1α/CXCR4 axis might involve in vein graft neointimal hyperplasia and subsequent atherosclerosis, which implicated SDF-1α/CXCR4 axis might be a valuable target for clinical interventions aimed at preventing vein graft diseases.

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