Fibroblast-specific protein 1 (FSP-1) plays multiple roles in promoting cell proliferation and motility. On creation of a vein graft, there was rapid deposition of platelets on the denuded surface leading to secretion of the chemokine stromal cell–derived factor-1α (SDF-1α). This was followed by recruitment of BM-derived cells expressing the SDF-1α receptor CXCR4; homing of FSP-1–positive cells was found to be dependent on platelet-derived SDF-1α. FSP-1 was expressed in 8% of the BM cells, and 20% of these express CD45; 85% of FSP-1–positive cells express CD11b. We found that the FSP-1–positive cells migrated into the vein graft in a Rac-1–dependent fashion. FSP-1 expression was also found to stimulate proliferation of SMCs through an MEK5-ERK5 signaling pathway that can be suppressed by a dominant-negative Rac1. Consequently, knocking down FSP-1 expression in BM cells prevented neointimal formation.

Conclusions: BM-derived FSP-1+ cells enhance neointima formation through an increase in transendothelial invasion with stimulation of SMC proliferation. The Rac1 and ERK5 signaling cascade mediate FSP-1–induced responses in SMCs and BM cells. This novel pathophysiology suggests a new therapeutic target, FSP-1, for preventing the development of neointima in vein grafts. (Circ Res. 2012;110:00-00.)

Key Words: fibroblast-specific protein 1 ▪ neointima formation ▪ platelets ▪ bone marrow ▪ smooth muscle cells

Arteriovenous (AV) fistula or vein grafts used in bypass surgery are commonly created to relieve angina or function as an access for hemodialysis. Failure of these grafts is characterized by hyperplasia of venous smooth muscle cells (VSMCs), neointima formation, and atherosclerosis. Several processes including endothelial dysfunction, neointima cell hyperplasia, and atherosclerosis are present in failed vein grafts. The pathogenesis of this lesion is incompletely understood. Identifying the pathogenesis of intimal hyperplasia in vein grafts could lead to strategies for facilitating the regression of lesions or reducing the likelihood of developing new lesions.

Neointima cells are derived from local VSMCs, adventitial fibroblasts, stem cells, or bone marrow (BM)-derived progenitors. In AV grafts, BM-derived, c-Kit–positive cells can assume the phenotype of either VSMC or endothelial cells, as determined by the expression of calponin or endothelial nitric oxide synthase, respectively. BM-derived cells can also assume other roles including conversion into inflammatory or progenitor cells that participate in the formation of neointima in AV grafts. A signal affecting the recruitment of BM cells during the development of the neointima could be SDF-1α and its receptor, CXCR4.

Fibroblast-specific protein (FSP-1, S100A4) is a member of the S100 family of calcium-binding proteins. It was isolated from mouse mammary adenocarcinoma cells, and its mRNA is detectable in cells of the BM, spleen, thymus, and lymphocytes. FSP-1 functions to regulate cellular motility through a direct interaction with myosin-IIA; FSP-1 also is associated with cancer cell metastasis, including invasive growth of mouse endothelial cells or neurons. How FSP-1 might affect the formation of neointima in vein grafts is not defined.

We show that BM-derived FSP-1+ cells are major components of remodeled AV grafts. BM-derived, FSP-1+ cells are attracted to the graft through an SDF-1α–dependent mecha-
ness and induce transendothelial invasion of inflammatory cells into the neointima. We also find that secreted FSP-1 influences the proliferation of VSMCs in AV grafts. These results provide new insights into the mechanisms by which FSP-1 BM-derived cells influence the formation of neointima.

Methods

An expanded Methods section is available in the Data Supplement at http://circres.ahajournals.org.

Mice and Vein Graft Procedure

All animal protocols were approved by IACUC. Wild-type (WT) C57/B6 mice aged 3 months were purchased from Jackson Laboratory (Bar Harbor, ME). FSP-1–GFP mice expressing the green fluorescent protein (GFP) under the control of the FSP-1 promoter were a generous gift from E.G., Neilson, Vanderbilt University School of Medicine. The vein graft procedure was performed as we previously described.13 See detailed information in the Online Methods section.

Reagents and Virus

Penicillin, streptomycin, DMEM, and FBS were obtained from Invitrogen Life Technologies (Carlsbad, CA). The protein assay kit was purchased from Bio-Rad (Hercules, CA). Recombinant adenovirus was kindly provided by Dr T.C. He (University of Chicago); the MEK5 dominant-negative vector was used as described.15 The MEK5 dominant-negative vector was used as an internal standard. Primers used were mouse FSP-1: forward 5'-TTCCAGAAGGTGATGAG-3'; reverse 5'-TCTATGGC AATGCGACAGAAGA-3'; and GAPDH forward 5'-AGTGGAGTGGC TGTT GAAATC-3', reverse 5'-TGCTGAGTATGTCGTGGAGT-3'.

Real-Time RT-PCR

Total RNA from freshly removed vena cava from mice or from vein grafts was isolated using the RNeasy kit (Qiagen, Valencia, CA). Real-time RT-PCR was performed using the Opticon real-time RT-PCR machine (MJ Research, Waltham, MA). The specificity of real-time RT-PCR was confirmed through agarose gel electrophoresis and melting-curve analysis. GAPDH was used as an internal control.

Cell Culture and Transfection

VSMCs were isolated from the inferior vena cava of WT mice and cultured as monolayers in DMEM supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 100 μg/mL penicillin, and 100 μg/mL streptomycin. The identity of VSMCs was determined by immunostaining with SMA-α actin antibody. The VSMC transfection (for knockdown ERK5) was performed by using program U23 with Nucleofector (Lonza, Basel, Switzerland). Cell proliferation assay was performed with XT kit (Sigma-Aldrich), following the manufacturer’s instructions.

Western Blot Analysis and Immunoprecipitation

Cells were lysed in RIPA buffer, and ~30 μg of proteins were separated by SDS-PAGE. After transferring to PVDF membranes, antibodies were added. To deplete FSP-1 from conditioned media, it was treated with anti-FSP-1 antibodies (2 μg/mL) for 2 hours before protein A/G agarose beads (Santa Cruz, CA) were added; the mixture was incubated overnight at 4°C. After centrifugation, FSP-1–depleted supernatants were collected.

BM Isolation and Transplantation

BM cells were harvested by flushing femurs and tibias of donor mice. The transplant was created by injecting 5 × 10⁶ BM cells into the lateral tail vein of lethally irradiated (1100 rads) mouse recipients.

Lentivirus Transduction to Knockdown FSP-1 in BM Cells

The short hairpin RNA of FSP-1 in lentivirus particles or control lentivirus particles expressing GFP only (Sigma Aldrich Inc) were incubated with BM cells from male WT mice. Lentivirus transductions of BM cells were performed over 12 hours in serum-free StemSpan TM SFEM media (Stem Cell Technologies), in the presence of cytokines (20 ng/mL IL-3, 100 ng/mL IL-6, and 100 ng/mL SCF, R & D Biosynthesis). Transduction efficiencies were determined by an analysis of GFP expression produced by control lentivirus particles. Red blood cells were lysed with an ammonium chloride lysis buffer (150 mmol/L NH₄Cl, 10 mmol/L KHCO₃, 0.1 mmol/L EDTA, pH 7.4).

Coculture and Conditioned Media-Induced Responses

FSP-1–GFP⁺ BM cells were isolated by cell sorting and about 2 × 10⁶ purified cells were cultured in the upper chamber of 24-well Boyden transwell plates (BD Biosciences, San Jose, CA) in DMEM supplemented with 10% FBS. After 48 hours, 5 × 10⁴ primary venous SMCs were placed in the cover slip in the lower transwell and examined with/without added BM cells. After 24 hours, BrdU was added for 45 minutes before fixing VSMCs. BrdU immunostaining was determined according to the manufacturer’s protocol (Roche

**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AV</td>
<td>arteriovenous</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>FSP-1</td>
<td>fibroblast-specific protein 1</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>stromal cell–derived factor 1 alpha</td>
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<tr>
<td>VSMCs</td>
<td>venous smooth muscle cells</td>
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Transendothelial Migration and Invasion Assay

Monolayers of $2 \times 10^6$ HUVEC were seeded in 24-well Boyden Transwell inserts for 24 hours and formed a confluent monolayer at 37°C in EGM2 media plus SingleQuots (Cambrex Bio Science, Walkersville, Inc). Inserts were placed over 24-well plates coated with a thin layer of 1% agarose in 1× PBS to facilitate removal of transmigrated cells for further analysis. Freshly isolated BM cells (2×10^6) from WT or FSP-1–GFP mice were added to the top chamber and allowed to transmigrate through HUVEC at 37°C. After 3 or 24 hours, the top chamber was removed and the cell number in the bottom chamber was determined using a fluorescent microscope. Each experiment was repeated at least 3 times to confirm our results. To determine the role of Rac-1 on BM cell transmigration, BM cells (5 million) were electroporated with or without dominant-negative Rac N17 expression plasmid (5 μg) by Neon system (Invitrogen); the working condition was set as voltage at 1650 V, 10 mm for 2 pulses.

Results

FSP-1 Expression in AV Grafts

Vein grafts were created in FSP-1–GFP^+ mice that expressed GFP under the control of the FSP-1 promoter. Histologically, remodeled vein grafts in FSP-1–GFP^+ mice were similar to those in WT mice. Specifically, in vena cavas from FSP-1–GFP mice, there were no FSP-1^+ cells, but 1 month after creation of the vein grafts, approximately one-third of the cells in vein grafts were FSP-1 positive (Figure 1A). Most FSP-1^+ cells were located in the adventitia, but they also were present in the neointima and endothelial layers (Figure 1A). Results from Western blots and real-time PCR confirmed the increase in FSP-1 expression in vein grafts after 1 month (42±7.2-fold compared with FSP-1 in isolated vena cava; Figure 1B and 1C). GFP staining in vein grafts placed in FSP-1–GFP^+ mice colocalized with FSP-1 (Figure 1D); no GFP staining was found in vein grafts placed in WT mice (data not shown).

Accumulation of BM-Derived FSP-1^+ Cells in Vein Grafts

To explore the origin of FSP-1^+ cells present in AV grafts, we created AV grafts using donor vena cava from FSP-1–GFP^+ or WT mice and placing them in WT or FSP-1–GFP^+ mice, respectively. GFP^+ cells were only found in AV grafts created in FSP-1–GFP^+ mice using veins from WT mice (Figure 2A). GFP^+ cells were absent in grafts created in WT mice using vena cava taken from FSP-1–GFP^+ mice (Figure 2B). Thus, FSP-1^+ cells did not arise from donor vena cava. GFP^+ cells were also found in AV grafts created in lethally irradiated WT mice that had BM transplants from FSP-1–GFP^+ mice (Figure 2D); a finding not present in AV grafts in WT mice transplanted with WT BM cells (Figure 2C). Thus FSP-1^+ cells found in AV grafts originate from BM cells.

BM-Derived FSP-1 Cells Express Markers of Inflammatory Cells in Vein Grafts

Using flow cytometry, we found no detectable FSP-1 expression in cells of either arteries or vena cava freshly isolated from WT mice (Online Figure I, A). However, ≈8% of BM cells were FSP-1 positive and 85.0±6.2% of the FSP-1–positive cells also stained positively for CD11b and 20.3±1.7% were CD45 positive (Figure 3A and 3B). Notably, GFP^+ BM cells from FSP-1–GFP mice also stained...
positively for collagen I and fibronectin but not for collagen III or SMA-α (Online Figure I, B and C). In vein grafts, FSP-1+ cells costained positively for CD45 and CD11b (Online Figure III, C and D). Most FSP-1+ cells did not costain positively with F4/80 (Online Figure I, D) in vein grafts after 1 month.

**Stromal Cell–Derived Factor 1α From Platelets Attracts FSP-1+ Cells Into Vein Grafts**

In donor vena cavas placed in aorta, endothelial cells were lost over the initial 24 hours after creating the AV graft. These cells were not replaced by 72 hours (Figure 4A). Instead, the exposed subendothelium was covered with activated platelets that stained positively for the platelet marker, CD41 (Online Figure II). At 5 days after creating the AV graft, there were FSP-1+ cells surrounded by platelets (Figure 4B). Notably, SDF-1α is secreted by activated platelets and functions as a chemoattractant for BM-derived progenitor cells. Therefore, we evaluated SDF-1α expression. It was detected within days of creating the vein graft and disappeared after 1 month (Figure 4C). CD41+ platelets were aggregated on the vein graft lacking endothelial cells and stained positively for SDF-1α (Figure 4D). Notably, some cells in the grafted vein coexpressed FSP-1 and CXCR4 (Figure 4E), suggesting that SDF-1α that is secreted by platelets attracts FSP-1+ cells into AV grafts and that these cells can respond to SDF-1α through its receptor, CXCR4.

**Transendothelial Migration and Invasive Properties of BM FSP-1+ cells**

In evaluating the function of FSP-1 cells from the BM, we found that transmigration of BM cells across an endothelial cell layer was significantly blocked when FSP-1 was knocked down in BM cells (Figure 5A). Likewise, pretreatment of BM cells with anti-CXCR4 antibodies led to a marked decrease in the transmigration potential of BM cells (Figure 5A). The percentage of GFP+ cells (ie, from FSP-1-GFP transgenic mice) that transmigrated increased significantly (47.6.3%) when compared with only 8.1.3% of GFP+ cells in BM of FSP-1–GFP mice (Figure 5B). Addition of SDF-1α increased the transmigration of GFP+ BM cells; this response was dramatically suppressed in BM cells that had been treated with FSP-1 shRNA or incubated with CXCR4 antibodies (Figure 5B). Treatment of BM cells to knockdown FSP-1 or addition of anti-CXCR4 antibodies to the BM cells also reduced the invasion of BM cells into 3D matrix gels, even though SDF-1α was present (Figure 5C). Furthermore, we found that increased invasion of the GFP+BM cells into 3D collagen gels was much higher compared with invasion of the GFP–BM cells (Figure 5D). To determine the underlying mechanism for FSP-1–mediated migration, we evaluated the role of small G-protein, Rac1, because its activity is related with cell migration; we found that overexpression of FSP-1 stimulated PAK-bound Rac1 activity (Figure 5E) and enhanced Rac1 membrane translocation (Figure 5F) in BM cells. Finally, FSP-1–stimulated BM-cell transendothelial migration was markedly blocked by dominant-negative Rac1 expression (Figure 5G).

**Expression of FSP-1 Induces VSMC Proliferation**

FSP-1+ cells in an AV graft were present in neointima areas that also exhibited PCNA+ proliferating cells (Figure 6A). To explore whether FSP-1+ cells could be involved in stimulat-
ing VSMCs hyperplasia, we isolated BM-derived FSP-1+ cells by sorting GFP+ cells from BM of FSP-1–GFP mice. Coculture of these GFP+ BM cells with VSMCs isolated from vena cava of WT mice led to proliferation of the VSMCs (Figure 6C). The link FSP-1 with proliferation was strengthened by our finding that lentivirus-mediated knockdown of FSP-1 in BM cells (Figure 6B) abolished its effect on VSMC proliferation (Figure 6C). Moreover, we found that conditioned media from BM cells isolated from FSP-1–GFP mice stimulated the growth of VSMCs, whereas the conditioned media from BM cells expressing shRNA to FSP-1 failed to stimulate VSMC proliferation (Figure 6D). Finally, depletion of FSP-1 from the conditioned media with a FSP-1 antibody significantly attenuated VSMC proliferation induced by conditioned media of FSP-1–GFP+ BM cells (Figure 6E).

**FSP-1 Stimulates VSMC Proliferation Through Activating Rac1 and ERK5**

We found that VSMCs exposed to the recombinant FSP-1 protein exhibited increased PCNA expression and BrdU incorporation in VSMCs (Figure 7A and 7B). To determine FSP-1–stimulated signaling responsible for this event, we found that FSP-1 significantly stimulated ERK5 phosphorylation; the earliest stimulation was observed at 5 minutes and peaked at 30 minutes (Figure 7C). FSP-1 also dose-dependently stimulated ERK5 phosphorylation (Online Figure III). In contrast, Treatment with FSP-1 has no significant effect on pAKT and pJNK levels (Figure 7C). Moreover, we found that inhibition of ERK5 by transfecting VSMCs with ERK5 siRNA or dominant-negative MEK5 suppressed FSP-1–induced expression of cell proliferation marker PCNA and cyclin D1 (Figure 7D and 7E). Similar responses were found in cell growth curve analysis (Figure 7F). Therefore, our results showed that both Rac1 and ERK5 were activated by FSP-1 and were critical for FSP-1–mediated cell migration and proliferation. To elucidate the sequence of activation of these molecules by FSP-1, we measured ERK5 and Rac1 activation when one of molecules was inhibited. The expression of dominant-negative Rac1(N17) inhibited the FSP-1–induced phosphorylation of MEK5 and ERK5 (Figure 7G) and proliferation (Figure 7H) in VSMCs. Although the expression of dominant-negative MEK5 blocked phosphorylation of ERK5, it had no effect on FSP-1–stimulated Rac1 activation (Figure 7I) and could not block FSP-1–induced BM cell transendothelial migration (data not shown).
results suggest that Rac1 acts upstream of MEK5/ERK5 in response to FSP-1.

**BM Transplantation With FSP-1 Knockdown Cells Suppresses Neointima Formation and VSMC Proliferation in AV Grafts**

To determine the role of FSP-1 in vivo, WT mice that had received a BM transplant with BM cells in which FSP-1 had been knocked down, there was downregulation of FSP-1 expression (data not shown). In vein grafts that were created in WT mice that had been transplanted with knockdown FSP-1–BM, the function of the AV graft (measured as the lumen-to-neointima ratio) was significantly improved (Figure 8A through 8C). AV grafts in these mice also had fewer FSP-1^+ cells as well as reduced SMA-α expression (Figure 8D and 8E). PCNA^+ proliferative cells were decreased in AV grafts in mice transplanted with FSP-1–BM cells (Figure 8F and 8G).

**Discussion**

FSP-1 (also known as S100A4, mts1, p9Ka, and metastasin) can mediate cellular proliferation, including the proliferation of VSMCs.21,22 FSP-1 was first cloned from serum-stimulated murine fibroblasts,23 and its overexpression was associated with neointima developing in the pulmonary artery; it also was found to be expressed in plexogenic arteriopathy of humans.24 These associations with neointima are similar to those we have uncovered (Figure 1).

FSP-1 has been implicated in controlling cell motility, possibly through the formation of complexes with a number of intracellular proteins (eg, the heavy chain of nonmuscle myosin II,9,25 liprin β1,26 and p53).27 Moreover, cancer cells expressing FSP-1 can exhibit aggressive migration properties with upregulation of matrix metalloproteinase 9,28 raising the possibility that FSP-1 could influence the migration of cells to form a neointima. For example, FSP-1 transgenic mice will form neointima in pulmonary arteries.21,29 Our data indicate that FSP-1 is expressed in BM-derived inflammatory cells (Figures 2 and 3), suggesting that FSP-1 may influence the motility of inflammatory cells infiltrating AV grafts. Interestingly, only a small fraction (8 ± 1.3%) of BM cells are FSP-1^+ cells as well as reduced SMA-α expression (Figure 8D and 8E). PCNA^+ proliferative cells were decreased in AV grafts in mice transplanted with FSP-1^+ BM cells (Figure 8F and 8G).

**Figure 4. Platelet secretion of SDF-1α leads to FSP-1^+ cells homing to AV grafts.** A, At 24 hours after creating the AV graft, the endothelium was denuded as detected by the staining of PECAM (red). Nuclei were stained by DAPI. B, FSP-1^+ (red) and CD41 (green) cells were found on the lumen surface at 5 days after AV grafts. C, AV grafts collected at different times were stained for SDF-1α and DAPI. D, Double staining with the platelet marker CD41 (red), and SDF-1α (green) indicates that SDF-1α released from CD41^+ cells. E, Double staining of FSP-1 (red) and the SDF-1α receptor CXCR4 (green) suggests that SDF-1α recruits FSP-1–positive cells. FSP-1^+ cells homing to AV grafts was associated with platelet-produced SDF-1α.
The hallmark of AV graft failure is neointima formation characterized by VSMC proliferation. Our results demonstrate that BM-derived FSP-1/H11001 cells accumulate in the AV graft, suggesting that they participate in the formation of a neointima. More concrete evidence of their role is that transplantation of the BM cells treated to knockdown FSP-1 expression significantly suppresses neointima formation (Figure 8). Thus, the actions of FSP-1 cells are important in mediating the dysfunction of AV grafts. FSP-1-mediated responses could involve at least 2 pathways: (1) an increase in transendothelial migration and collagen gel invasion of FSP-1 inflammatory cells and (2) local paracrine stimulation of VSMC proliferation in AV grafts. In fact, FSP-1 is known to be secreted, and our results suggest it acts in a paracrine fashion to increase proliferation of VSMC. Specifically, we demonstrated that coculture of FSP-1 BM cells stimulates the growth of VSMC (Figure 6). We also found that VSMC proliferation was blocked by inhibition of MEK5/ERK5 signaling pathway, suggesting that FSP-1 stimulates proliferation through ERK5 activation (Figure 7). ERK5 has been linked with cell proliferation and differentiation. We found that FSP-1 stimulated ERK5 phosphorylation in a time- and dose-dependent manner. Furthermore, we found that Rac1 can regulate FSP-1-induced MEK5/ERK5 phosphorylation and VSMC proliferation (Figure 7G and 7H). Similar responses have been reported that Rac1 involved in regulating MAPK activation through p21-activated kinases or JNK/AP1 pathway. In contrast, FSP-1–stimulated Rac1 activation could not be inhibited by negative dominant MEK5, suggesting that Rac1 acts as an upstream of ERK5. FSP-1 signaling could be stimulated by activating the receptor for advanced glycation end products (RAGE). In this formulation, FSP-1 proteins (ie, S100 proteins), released from inflammatory cells, could activate RAGE and induce proinflammatory adhesion molecules as well as cytokines, tissue-destroying elastases, and MMPs. Serotonin has been shown to stimulate the release of FSP-1 from SMCs, and FSP-1 can bind with RAGE and induce migration of SMCs through an intracellular chloride channel. Oligomerization of S100 proteins appears to be essential for RAGE-mediated stimula-
tion of MMP in chondrocytes. RAGE also mediates S100A4-stimulated MMP13 release. We found that platelets (stained positively for CD41, the platelet marker) were deposited in the denuded AV graft (Figure 4). Platelets also expressed SDF-1, which is released from apoptotic VSMCs and platelets. Because adhesion of platelet to the exposed subendothelium is the first response to vascular injury, we explored the involvement of MMP in chondrocytes. RAGE also mediates S100A4-stimulated MMP13 release.

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Figure 6. FSP-1 induces VSMC proliferation. A, at 1 month after creating the AV graft, serial slides of the vein grafts were examined. FSP-1–positive cells were stained with SMA-α antibodies (left panel); proliferation was assessed by PCNA staining (right panel). B, BM cells infected with FSP-1 shRNA lentivirus for 24 hours had reduced the expression of FSP-1, even though BM FSP-1+ cells had been exposed to PDGF-BB for 24 hours. C, VSMCs were cocultured with BM cells that had been treated to knockdown FSP-1. In 3 different experiments, there was a decrease in BrdU-positive cells counted in 5 different areas. D, Conditioned-media (CM) from BM FSP-1+ cells induced VSMC proliferation as measured by BrdU incorporation. This response was blocked by adding CM from BM cells that had been treated to knockdown FSP-1. E, CM from FSP-1+ cells was incubated with control or anti–FSP-1 antibodies and subsequently immunoprecipitated with agarose-A/G to remove FSP-1. VSMCs treated with FSP-1-conditioned media were subjected to BrdU analysis.

Figure 7. FSP-1 stimulates VSMC proliferation and migration through Rac1-dependent pathway. A and B, After 48 hours of exposure to recombinant FSP-1, VSMC proliferation increased as detected by PCNA expression (A) and BrdU incorporation (B). C, FSP-1 stimulated ERK5 phosphorylation. VSMCs were treated with FSP-1 (100 ng/mL) after serum starvation for 24 hours; cell lysates were collected, and the phosphorylation of ERK5, ERK1/2, p38, JNK, and AKT were detected by Western blots. GAPDH was used as a loading control. D, ERK5 siRNA knocked down ERK5 expression in VSMCs. E and F, FSP-1–induced cell proliferation was inhibited by blocking MEK5/ERK5 signaling pathway. VSMCs were transfected with ERK5 siRNA or MEK5 dominant-negative plasmids; the FSP-1–induced proliferative markers were detected by Western blot (E); and the XTT assay was used to measure the cell proliferation (F). The effects of FSP-1 on MEK5/ERK5 phosphorylation (G) and proliferation (H) were suppressed by dominant-negative Rac1 expression plasmids in VSMCs. I, The expression of dominant-negative MEK5 had no effect on FSP-1–induced Rac1 activation.
the SDF-1α/CXCR4 axis in the recruitment of BM-derived FSP-1+ cells into the AV graft. SDF-1α expression was increased in AV grafts. In denuded veins of the AV graft, we found cells expressing both FSP-1 and the SDF-1α receptor, CXCR4 (Figure 4). Because SDF-1α expression can mobilize and recruit peripheral blood progenitor cells into injured vessels,41 our results suggest that the recruitment of FSP-1+ cells occurs when platelets are deposited on the denuded vena cava of the AV graft.

It is widely accepted that neointima formation involves migration and proliferation of adjacent cells. These also is, however, accumulating evidence that cells from the BM give rise to endothelial cells and VSMCs, which contribute to vascular healing, remodeling, and neointima formation.42 Because BM cells can be directly involved in vascular remodeling, strategies to prevent neointima formation have been based on genetically engineering BM cells. For example, knockout of ROCK-143 or ASC44 (apoptosis-associated speck-like protein containing a caspase recruitment domain) in BM cells was found to decrease neointima formation after carotid artery ligation. Moreover, we found that knockdown FSP-1 in BM cells will decrease the accumulation of inflammatory cells and the formation of neointima in AV grafts (Figure 8).

We conclude that after creation of an AV graft, the endothelial layer cell layer is rapidly lost, and platelets cover the subendothelium. SDF-1α is secreted by these platelets to recruit BM-derived FSP-1+ cells into the vein graft. Cells expressing FSP-1 exhibit a higher Rac1 activation and an increased capacity for migration and invasion. This results in the development of inflammation from penetration and infiltration of FSP-1+ cells into the vein grafts. Secreted FSP-1 also exhibits a paracrine role, which stimulates MEK5-ERK5 signaling pathway through Rac1 activation to promote proliferation of surrounding VSMCs, leading to neointima formation.

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Disclosures
None.

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Novelty and Significance

**What Is Known?**
- Arteriovenous (AV) grafts fail because of neointima formation.
- Smooth muscle cell (SMC) proliferation is a major reason for intimal hyperplasia.
- In addition to SMCs, the neointima contains several other types of cells, including fibroblast specific protein 1 (FSP-1)–positive cells.

**What New Information Does This Article Contribute?**
- FSP-1–positive cells are recruited by a mechanism involving a platelet-secreted stromal cell–derived factor (SDF-1) and its receptor.
- FSP-1 promotes inflammatory cell infiltration into the subendothelial space in AV grafts. It also functions as paracrine growth factor stimulating SMC growth.

- FSP-1 knockdown in bone marrow cells inhibited neointima formation.

The hallmark of vein graft failure is neointima formation characterized by SMC accumulation, but the mechanism of cell accumulation in the neointima is poorly understood. In this study, we found that bone marrow–derived FSP-1–positive cells were recruited into the neointima by a mechanism involving a platelet-secreted stromal cell–derived factor and its receptor. Our results show that FSP-1 promotes inflammatory cell infiltration into neointima and that it functions as paracrine growth factor, which stimulates SMC growth. Ex vivo FSP-1 knockdown in bone marrow attenuated FSP-1–induced inflammatory cell infiltration, SMC proliferation, and neointima formation. These findings suggest that FSP-1 may have an obligatory role in vein graft failure.
FSP-1 Silencing in Bone Marrow Cells Suppresses Neointima Formation in Vein Graft
Jizhong Cheng, Yun Wang, Anlin Liang, Lixin Jia and Jie Du

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Supplemental Material

Methods

Vein Graft procedure. In brief, the right common carotid artery of a male mouse was mobilized and divided. A cuff was placed on both ends of the artery and the ends were everted over the cuff and ligated with an 8.0 silk ligature. Vena cava from donor mice were grafted between the 2 ends of the carotid artery by “sleeving” the ends of the vein over the artery cuff and secured with 8.0 silk sutures\(^1\). After 4 weeks, the vein grafts were obtained. Vessel wall thickness was measured as area of the vessel minus that of the lumen using NIS-Elements BR 3.0 program.

Immunohistochemistry. Sections were blocked with 10% goat serum (Vector Laboratories, Burlingame, CA) for 30 min and then incubated with primary antibodies (FSP-1, 1:500; SMA-\(\alpha\), 1:3000). Sections were washed in 0.5% Tween 20 in PBS (PBST) and incubated with a biotinylated secondary antibody (Vector lab) at room temperature\(^1\). After PBST washes, tissue sections were incubated with an Elite\(^{\text{®}}\) ABCreagent (Vector Laboratories) followed by a peroxidase substrate kit (Vector Laboratories) according to the manufacturer’s protocol. The sections were counterstained by hematoxylin. For double immunofluorescent staining of samples, fluorescent secondary antibodies were applied to sections; DAPI was used as counter staining. Pictures were recorded using a Nikon Eclipse 80i fluorescence microscope (Melville, NY) with a negative control as an isotype control IgG or PBST.
Supplemental Figure Legends:

Supplemental Figure I. Characterization of FSP-1\(^+\) cells. **A.** Flow cytometric analysis of FSP-1 expression in mouse tissues; cells from arteries, veins, hearts, and bone marrow from WT or FSP-1-GFP\(^+\) mice were collected to identify GFP\(^+\) cells. **B.** BM cells were isolated from FSP-1-GFP\(^+\) mice and incubated with antibodies to cell markers CD3, B220, leukocyte CD45, and mononuclear cells CD11b before flow cytometry analysis. **C.** FSP-1-GFP\(^+\) BM cells were cultured on cover slips and stained with different antibodies: FSP-1\(^+\) cells in red, GFP\(^+\) cells in green and nuclei shown in blue. **D.** Characterization of FSP-1\(^+\) cells in vein grafts. Co-staining of vein grafts for FSP-1 and macrophage marker F4/80.

Supplemental Figure II. Platelets accumulation in AV grafts. CD41\(^+\) platelets accumulated on the lumen surface at 5 days after creation of AV grafts.

Supplemental Figure III. Recombinant FSP-1 stimulates ERK5 phosphorylation in VSMCs. VSMCs were treated with different doses of FSP-1 for 30 min after starvation for 24 h, cell lysates were collected and the phosphorylated ERK5, ERK1/2, p38, JNK and AKT were detected by Western blots. GAPDH was used as a loading control.

Reference List

Supplemental Figure I
Supplemental Figure II

400X

CD41  DAPI  Merge
Supplemental Figure III

FSP-1 (ng/ml)  |  0  |  10  |  50  |  100 |  500 |
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