Activation Transcription Factor-4 Induced by Fibroblast Growth Factor-2 Regulates Vascular Endothelial Growth Factor-A Transcription in Vascular Smooth Muscle Cells and Mediates Intimal Thickening in Rat Arteries Following Balloon Injury

Kristine P. Malabanan, Peter Kanellakis, Alexander Bobik, Levon M. Khachigian

Abstract—Activation transcription factor (ATF)-4 is a member of the ATF/CREB family of basic leucine zipper transcription factors that regulates cellular responses to a variety of stresses. The role of ATF-4 in smooth muscle cells of the vessel wall is completely unknown. Here, we show that ATF-4 expression is induced in smooth muscle cells in response to injury, both in vitro using a model of mechanical injury and in the media of balloon-injured rat carotid arteries. We demonstrate that ATF-4 is activated by fibroblast growth factor (FGF)-2, an injury-induced mitogen, through the phosphatidylinositol 3-kinase pathway. Injury also activates vascular endothelial growth factor (VEGF)-A, whose expression is stimulated by ATF-4 overexpression and exposure to FGF-2. FGF-2 induces ATF-4 binding to a recognition element located in the VEGF-A gene at +1767 bp and luciferase reporter gene expression dependent on this site. Moreover, ATF-4 knockdown with small interfering RNA or ATF-4 deficiency ameliorates FGF-2–inducible VEGF-A expression. Intraluminal delivery of ATF-4 small interfering RNA in rat carotid arteries blocks balloon injury–inducible ATF-4 and VEGF-A expression after 4 hours and intimal thickening after 14 days. These findings reveal, for the first time, the induction of ATF-4 by both vascular injury and FGF-2. ATF-4 serves as a conduit for the inducible expression of 1 growth factor by another during the process of intimal thickening. (Circ Res. 2008;103:378-387.)

Key Words: ATF-4 ■ FGF-2 ■ VEGF-A ■ intimal thickening

Injury to the artery wall triggers a complex sequence of cellular events, including the accumulation of smooth muscle cells (SMCs) in the intima. SMC growth contributes to the formation of the lesion and loss of lumen diameter and vascular contractility, which underpin such pathologies as atherosclerosis and restenosis after balloon angioplasty. At the molecular level, these events are initiated and maintained by transcription factors. Activation of transcription factors can result in the altered expression of pathophysiologically relevant genes, making key transcription factors potential therapeutic targets. Numerous transcription factors are induced in the vascular SMC response to injury, including nuclear factor κB, E2F, c-Jun, and Egr-1. A more complete understanding of the transcription factors regulating the response to injury would lead to more effective strategies to control vascular occlusive disorders.

Activation transcription factor (ATF)-4 is a bZIP (basic leucine zipper domain) transcription factor that belongs to the cAMP-responsive element binding (CREB) protein family. Able to form heterodimers with members of the AP-1 and C/EBP family of proteins, ATF-4 (also known as CREB2, TAXREB67, C/ATF) can act either as an activator or as a repressor. ATF-4 is a regulator of pathways through which mammalian cells respond to amino acid deficiency. It controls genes involved in amino acid import, glutathione biosynthesis, and resistance to oxidative stress. Consequently, transcriptional upregulation of ATF-4 has been demonstrated in a variety of contexts: by nitric oxide in human monocytes, by homocysteine in human vascular endothelial cells, by superoxide in ischemic neurons, and by heregulin, a combinatorial ligand for human epidermal growth factor receptor-3 and for human epidermal growth factor receptor-4, in breast cancer cells. However, the expression of ATF-4 in the artery wall and its possible involvement in the response to injury have not yet been characterized.

In efforts to identify new transcription factors involved in the process of neointima formation, here, we used microarray studies to screen for key genes differentially expressed by rat aortic SMCs treated with fibroblast growth factor (FGF)-2, a
growth factor that is released by injured vascular cells in vitro and in vivo\textsuperscript{12,13} and that has long been implicated in the pathogenesis of atherosclerosis and restenosis.\textsuperscript{14} We demonstrate ATF-4 induction by FGF-2 in vascular SMCs in vitro and in the mediad compartment of balloon-injured rat carotid arteries. Moreover, we show that ATF-4 plays a critical role in the transcriptional induction of vascular endothelial growth factor (VEGF)-A, which is itself activated by both FGF-2\textsuperscript{15,16} and balloon injury.\textsuperscript{17} We propose that ATF-4 serves as a key conduit for the injury-inducible expression of VEGF-A by FGF-2 in the reparative response to injury.

**Materials and Methods**

Details on cell culture, plasmid construction and transient transfection, luciferase assays, RT-PCR and quantitative (Q)RT-PCR, immunoprecipitation, Western blotting, EMSA, SMC proliferation, the carotid artery injury, chromatin immunoprecipitation (ChIP), and immunohistochemistry can be found in the online data supplement at http://circres.ahajournals.org.

**Results and Discussion**

**ATF-4 Is Inducibly Expressed by Vascular SMCs in Response to Injury, In Vivo and In Vitro**

Immunohistochemical analysis of uninjured rat carotid arteries revealed that ATF-4 is poorly expressed in medial SMCs. However, ATF-4 expression was readily detectable in these SMCs within 4 hours of injury (Figure 1A). To determine whether ATF-4 is expressed at longer time points after balloon injury, we stained the arteries for ATF-4, 5 days (when the neointima was starting to become visible) and 14 days (when an extensive neointima had formed) after balloon injury. ATF-4 staining was apparent in both the media and intima at these later time points, although staining intensity was considerably less intense compared to 4 hours postinjury (Figure 1A). Immunochemical analysis at all time points was performed simultaneously to avoid inconsistencies in staining and any difficulties interpreting the data.

In support of these data, we injured cultured rat aortic SMCs in vitro using a well-established scraping model.\textsuperscript{18,19} RT-PCR analysis revealed that ATF-4 transcript levels increased after injury in a time-dependent manner (Figure 1B). The inducible expression of ATF-4 in this model followed that of the immediate early gene, early growth response-1 (Egr-1), which increased within 30 minutes (Figure 1B).\textsuperscript{20} QRT-PCR analysis revealed that ATF-4 transcript levels increased 2.5-fold within 2 hours of in vitro injury (Figure 1C). This is the first demonstration of the induction of ATF-4 by injury in vascular SMCs.
FGF-2 Stimulates ATF-4 Expression in Vascular SMCs

We next examined whether ATF-4 can be induced by FGF-2, because we and others have previously shown that injury triggers the rapid release of endogenous FGF-2 from SMCs. RT-PCR analysis of FGF-2–treated SMCs revealed increased ATF-4 expression within 1 to 2 hours of exposure to FGF-2, which remained elevated at 4 hours (Figure 2A). Real-time PCR analysis likewise demonstrated increased ATF-4 mRNA levels, reaching 2.5-fold after 4 hours (Figure 2B). Immuno-precipitation of ATF-4 from lysates of FGF-2–treated SMCs followed by immunoblotting for ATF-4 revealed increased levels of ATF-4 protein within 2 hours of growth factor exposure (Figure 2C, left). Substituting ATF-4 antibodies (rabbit polyclonal IgG isotype) with species- and isotype-matched antibodies in the immunoprecipitation step to nuclear factor κB p65 did not give rise to an ATF-4 signal (Figure 2C, left). The induction of ATF-4 by FGF-2, like injury, has not been described previously in any cell type. ATF-4 expression was unchanged up to 4 hours after bovine aortic endothelial cells were exposed to FGF-2 or in vitro injury (data not shown), suggesting cell-restricted regulation of ATF-4 expression.

To provide confirmatory evidence linking ATF-4 with endoplasmic reticulum stress in SMCs, we performed Western blot analysis using extracts of SMCs various times after scraping injury or exposure to FGF-2. It is well established that endoplasmic reticulum stress activates PERK-dependent eIF2α phosphorylation and that this axis lies upstream of ATF-4 expression. Our data show that both injury and FGF-2 increase phosho-eIF2α levels within 1 hour (Figure 2C, right).

Pathways of ATF-4 Activation

To determine the signaling pathways through which FGF-2 activates ATF-4, we performed pharmacological inhibitors of 2 known kinase pathways were used 1 hour before 4 hours of treatment with FGF-2. QRT-PCR analysis revealed that the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 blocked FGF-2–dependent induction of ATF-4. In contrast, the extracellular signal-regulated kinase (ERK) inhibitor PD98059 had no effect (Figure 3A, top). Western blot analysis for the phosphorylated form of Akt verified the activation of the PI3K pathway by FGF-2 and inhibition of this process by LY294002 (Figure 3A, bottom). Immunohistochemical staining confirmed that Akt phosphorylation is increased within 4 hours of injury (Figure 3B). Phospho-Akt levels remain elevated 5 and 14 days after injury (Figure 3B).

ATF-4 Overexpression Increases VEGF-A Levels

VEGF-A exacerbates the mitogenic effect of FGF-2 in the injured vessel wall. Pretreatment with VEGF-A has been
found to significantly increase intimal SMC replication in response to FGF-2 in rat carotid arteries, as compared to infusion with FGF-2 alone. To establish a regulatory role for ATF-4 as a mediator of FGF-2–inducible VEGF-A expression, the CMV-driven expression vector pcDNA3.1/ATF-4 (20 μg) was transfected into rat aortic SMCs, and the backbone (pcDNA3.1) was used as control. ATF-4 overexpression induced VEGF-A mRNA levels (Figure 4A). ATF-4 also increased the expression of numerous other genes that had been differentially expressed in the ATF-4/H11001/H11002 mouse embryonic fibroblast (MEF) screen, including platelet-derived growth factor (PDGF) receptor-α, cholesterol 25-hydroxylase, and angiotensin II receptor 2 (Figure 4A). On the other hand, PDGF-A levels remained unchanged (Figure 4A). We further observed a 4-fold increase in the expression of VEGF-164, the VEGF-A isoform predominantly expressed in SMCs, in ATF-4 transfectants as compared to the backbone alone (Figure 4B).

**FGF-2 Induces ATF-4 Binding to a Recognition Element Located in the VEGF-A Gene at +1767 bp and Luciferase Expression Dependent on This Site**

QRT-PCR analysis of FGF-2–treated SMCs shows that FGF-2 induced transcription of VEGF-A 8 hours after exposure to FGF-2 (Figure 5), consistent with the induction observed with ATF-4 (Figure 4). Previous studies have demonstrated the existence of a single functional ATF-4 binding site in the VEGF-A gene. A 32P-labeled double-stranded oligonucleotide spanning this element was used in electrophoretic mobility-shift assay (EMSA), together with extracts of SMCs exposed to FGF-2 for 2 hours. Incubation with FGF-2 induced nucleoprotein complex formation, which was competed for by a 100-fold excess of unlabeled Oligo VEGF-A1752/1786, but not by mOligo VEGF-A1752/1786, in which the ATF-4 recognition element was mutated (5’-GATTACATC-3’ to 5’-AATCATACA-3’). FGF-2–inducible nucleoprotein complex formation was also abolished by the presence of a molar excess of ATF-4 Oligo, an oligonucleotide bearing a consensus ATF-4 binding site (Figure 6A, top). We also used a commercial preparation of recombinant human ATF-4 protein in EMSA. The protein formed a complex with 32P-Oligo VEGF-A1752/1786, whereas 32P-mOligo VEGF-A1752/1786 failed to form this complex (Figure 6A, middle). We next performed the EMSA with nuclear extracts of SMCs 2 hours after scraping injury, when ATF-4 expression is clearly increased (Figure 1B). Injury-inducible nucleoprotein complex formation was abrogated by a molar excess of unlabeled Oligo VEGF-A1752/1786 or ATF Oligo, and 32P-mOligo VEGF-A1752/1786 did not form an inducible complex (Figure 6A, bottom). Extending these findings, we performed ChIP analysis with extracts of cells untreated or treated with FGF-2 for 2 hours. Figure 6B demonstrates that endogenous ATF-4 binds to the authentic VEGF-A gene in cells exposed to FGF-2, whereas the amplicon is not bound by p65 or YY1.

To determine whether FGF-inducible ATF-4 is able to transactivate from this site in the human VEGF-A gene, this nucleotide motif was cloned into pGL3prom, to create pGL3prom-VEGF-A1752/1786, pGL3prom-VEGF-Am1752/1786 (the oligonucleotide bearing the mutant sequence) and pGL3prom-VEGF-Arev1752/1786 (oligonucleotide in reverse orientation). These constructs were transfected into SMCs and then treated with FGF-2 for 24 hours. Luciferase assays revealed that FGF-2 stimulated expression from this element in pGL3prom-VEGF-A1752/1786 and pGL3prom-VEGF-Arev1752/1786 but not pGL3prom-VEGF-Am1752/1786 nor the backbone pGL3prom alone (Figure 6C). This complements the binding data and provides the first demonstration that ATF-4 mediates FGF-2–inducible reporter gene expression dependent on the VEGF-A ATF-4 element.
ATF-4 Deficiency Ameliorates FGF-2–Inducible VEGF-A Expression

To examine the effect of ATF-4 deficiency on FGF-2–inducible VEGF-A expression, we next used SV40-transformed embryonic fibroblasts derived from ATF-4/H11001/H11001 and ATF-4/H11002/H11002 mice. QRT-PCR analysis demonstrates a 4-fold reduction in VEGF-A transcript levels in ATF-4/H11002/H11002 MEFs as compared to the ATF-4/H11001/H11001 MEFs at all time points between 2 and 8 hours after exposure to FGF-2 (Figure 7A). In addition, small interfering (si)RNA targeting ATF-4 (0.4 μmol/L siRNA) blocked FGF-2 induction of ATF-4 expression after 4 hours in SMCs, whereas the scrambled siRNA counterpart failed to inhibit (Figure 7B). Moreover, the ATF-4 siRNA prevented FGF-inducible VEGF-A mRNA levels after 8 hours, compared to VEGF-A levels in untreated SMCs (Figure 7C).

Figure 4. ATF-4 overexpression induces VEGF-A transcription. A, CMV-driven expression vector pcDNA3.1/ATF-4 (20 μg) was transfected into SMCs, with the backbone alone (pcDNA3.1) used as control. Total RNA was isolated after 8 hours and used for RT-PCR analysis with primers directed against ATF-4, VEGF-A, PDGF receptor-α (PDGF-Rα), cholesterol 25-hydroxylase (Ch25h), angiotensin II receptor 2 (AT2R), and PDGF-A. GAPDH levels show unbiased loading. B, QRT-PCR analysis was carried out on the same samples, using primers for VEGF-A164. Data were normalized to GAPDH. *P<0.05.

ATF-4 siRNA Blocks Injury-Inducible VEGF-A Expression and Neointima Formation in Balloon-Injured Carotid Arteries

Finally, to determine whether ATF-4 regulates the process of intimal hyperplasia in response to injury, we balloon-injured rat carotid arteries,18,27 then infused ATF-4 siRNA (50 μg bolus) intraluminally for 20 minutes, and then performed immunohistochemistry 4 hours and 14 days after injury. ATF-4 siRNA virtually abrogated ATF-4 expression at 4 hours (Figure 8A), when levels of ATF-4 in control injured arteries are at their highest of all time points examined (Figure 1A). By 14 days postinjury, although ATF-4 expression is comparatively weaker (Figure 1A), the siRNA still reduced ATF-4 expression (Figure 8A). In contrast, ATF-4 immunostaining was not influenced by the control siRNA (ATF-4 siRNAsc), with scrambled sequence (Figure 8A). Also, importantly, VEGF-A induced by injury was blocked by the ATF-4 siRNA (Figure 8B). Morphometric analysis 14
days after balloon injury demonstrates a reduction in intimal thickening (Figure 8C and 8D). These data link VEGF-A with ATF-4 in balloon-injured arteries and demonstrate the dependency of VEGF-A expression on ATF-4 in the vessel. ATF-4 is necessary for intimal thickening in rat carotid arteries after vascular injury. We did not observe any significant mitogenic effect of VEGF-A (unlike FGF-2) on SMC growth after 3 days (Figure 8E, top), consistent with previous reports.28 ATF-4 overexpression, on the other hand, stimulated SMC proliferation after 3 days, and this growth was unaffected by the presence of neutralizing VEGF-A antibodies (Figure 8E, bottom). These data demonstrate that the mitogenic effect of ATF-4 on SMCs is mediated by factors other than VEGF-A.

This study provides the first demonstration of ATF-4 induction in vascular SMCs by arterial balloon injury in vivo and mechanical injury in vitro. It is also the first demonstra-
tion of the activation of ATF-4 mRNA, protein, and DNA-binding by FGF-2, an injury-induced growth factor, through the PI3K pathway. Because ATF-4 is associated with cellular stress and, in particular, endoplasmic reticulum stress, our findings are supported by previous reports that have linked endoplasmic reticulum stress with the development of atherosclerosis and ischemic heart disease. The present findings also demonstrate that ATF-4 is both necessary and sufficient to induce VEGF-A transcription; the absence of ATF-4 perturbs FGF-2–inducible VEGF-A transcription, in both vascular SMCs and in MEFs. VEGF-A expression by medial SMCs is suppressed when ATF-4 is silenced using siRNA.

VEGF-A is a secreted glycoprotein and a potent angiogenic factor, regulating embryonic, physiological, and pathological blood vessel growth in vivo. Although originally identified as an endothelial cell mitogen and vascular permeability factor, it is now clear that VEGF-A plays significant roles in other cell types and contexts. With at least 5 known isoforms, its expression is regulated at multiple levels, including transcription, in which AP-1, Sp1, Egr-1, and HIF-1 play a role. Like other growth regulatory genes, such as FGF-2 and PDGF-A, transcription of VEGF-A is made more complex by the presence of a long (1-kb) and G+C-rich 5′ untranslated region that carries secondary structure. It would not be surprising that elements outside the VEGF-A proximal promoter would play a role in regulating its transcription. Our results indicate that an element located +1767 bp in the VEGF-A gene is bound by ATF-4 in response to FGF-2 (Figure 6A, top, and 6B) and injury (Figure 6A, bottom) and confers responsiveness of a heterologous construct to FGF-2 (Figure 6C). Specificity of both binding and transactivation by ATF-4 is evidenced by ablation of this effect when the ATF-4 site was mutated. We have previously investigated growth factor regulation of another growth factor at the level of transcription. For example, Egr-1 mediates FGF-1–induced PDGF-A chain expression in endothelial cells. Egr-1 also controls increased PDGF-C transcription in SMCs exposed to FGF-2. PDGF-BB stimulates PDGF-A in SMCs via Ets-1 and Sp1.

FGF-2 is an important activator of gene expression programs in the injured artery wall because of its existence in a preformed state in uninjured arteries and its rapid release on injury. Our own work has shown that FGF-2 is released within 5 minutes in stented human coronary arteries, whereas transforming growth factor-β and P-selectin levels are unchanged. Interestingly, ATF-4 expression is induced by a number of factors other than FGF-2 that have been implicated in atherogenesis and intimal hyperplasia, such as nitric oxide, osteopontin, and homocysteine. It is, therefore, unlikely that FGF-2 is the only mediator of inducible ATF-4 expression. Future studies should determine whether ATF-4 functionally regulates the effects of injury-induced factors other than FGF-2 in the artery wall. Our studies also open up opportunities of exploring the role of ATF-4 in the regulation of pathophysiologically relevant genes besides VEGF-A. These include PDGF receptor-α, cholesterol 25-hydroxylase, and angiotensin II receptor 2. Consistent with this, there are numerous putative ATF-4 binding motifs in the promoters of these genes. Interestingly, ATF-4 increases PDGF receptor-α expression without affecting levels of PDGF-A. These findings broaden the scope of ATF-4 target genes beyond VEGF-A and implicate ATF-4 as a key regulator in SMC pathobiology. It will also be interesting to explore the functional interrelationship between ATF-4
and other transcription factors (such as Egr-1, c-Jun, and Ets-1) that are also induced by injury. The ATF-4 promoter contains several putative binding sites for Egr-1, c-Jun/AP-1, and Ets-1, suggesting that the expression of ATF-4 may be influenced by these SMC injury-inducible transcription factors. ATF-4 may also regulate these other transcription factors through protein:protein interactions. Although the capacity of ATF-4 to interact with Egr-1 and Ets-1 has not yet been reported, its ability to bind and functionally cooperate with other transcription factors is well established.

Figure 8. ATF-4 knockdown inhibits VEGF-A expression and neointima formation in balloon-injured rat carotid arteries. A, Rat carotid arteries were balloon-injured and perfused with ATF-4 siRNA or scrambled counterpart (50 μg). After 4 hours and 14 days, arteries were harvested and sections (5 μm) were immunostained for ATF-4. B, VEGF immunoreactivity in the balloon-injured vessels treated with ATF-4 siRNA or siRNAsc. Right, Lack of specific signal when the primary (1°) antibody is omitted. C, Representative micrographs of elastin-stained cross sections of vessels 14 days following injury and intraluminal delivery. Top, High-magnification (×400) representative micrographs delineating intima, media, and adventitia. Bottom, Low-magnification (×100) micrographs. D, Intimal thickening measured using a computer-interfaced imaging system. E, Effect of ATF-4 overexpression and VEGF-A addition on SMC proliferation. Top, Growth-quiescent SMCs were incubated with VEGF-A or FGF-2 (25, 50, or 100 ng/mL for each) for 72 hours before counting trypsinized cells with a Coulter counter. Bottom, Alternatively, SMCs were transfected with ATF-4-pcDNA3 (0.3 μg) and incubated with neutralizing antibodies to VEGF-A (VEGF-ANAb) or IgG for 72 hours and then counted. *P < 0.05.
The role of VEGF-A in vascular injury is poorly understood. VEGF-A mRNA and protein levels are increased in endothelium-denuded porcine arteries
 and in balloon-injured rabbit arteries.\(^4^9\) On one hand, some reports indicate that VEGF-A is an endothelial cell mitogen with poor, if any, positive influence on SMC proliferation.\(^2^8\) VEGF-A is thought to attenuate SMC hyperplasia by stimulating reendothelialization.\(^5^0,5^1\) On the other hand, several lines of evidence indicate that VEGF-A stimulates neointima formation. For example, local gene transfer of VEGF-A significantly increases intimal hyperplasia after balloon injury\(^4^9\) or application of silastic collars.\(^5^2\) Intraluminal infusion of VEGF-A to denuded rat carotid arteries doubled the mitogenic response to infused FGF-2 by increasing intimal SMC replication.\(^2^4\) Moreover, systemic delivery of soluble Flt1 (which blocks VEGF-A–VEGFR1/Flt1 binding) attenuated neointima formation after balloon injury without affecting luminal reendothelialization.\(^5^3\) The ability of VEGF-A to influence the behavior of nonendothelial cell types may be mediated by Flt-1, which is highly expressed in injured vessels.\(^2^4\) The VEGF-A–Flt-1 axis promotes the recruitment of SMCs and inflammatory cells,\(^5^4\) increasing matrix metalloproteinase production, which promote SMC migration.\(^5^5\) The present study does not intend to reconcile these apparently different paradigms. Instead, it integrates, for the first time, FGF-2 (which is well known to be released locally on acute vascular injury)\(^5^6\) with VEGF-A expression in SMCs\(^1^6\) through PI3K-dependent ATF-4 induction and VEGF-A transactivation. Our findings show that silencing ATF-4 inhibits injury-inducible VEGF-A expression and intimal hyperplasia in rat carotid arteries. This defines a new role for ATF-4 as a phenotypic regulator in the vascular response to injury.

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None.

References


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Cell Culture, Plasmid Construction and Transient Transfection

Primary rat aortic and human coronary artery SMCs were obtained from Cell Applications, Inc. (San Diego, CA) and cultured in Waymouth’s medium, pH7.4, containing 10% FBS, 10units/ml penicillin, and 10µg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. The cells were rendered quiescent by incubation in Waymouth’s medium, without foetal bovine serum for 24h. Cells were not used beyond passage 8 in experiments. Mouse embryonic fibroblasts (ATF-4 -/- and +/+ SV40 transformed) were obtained from David Ron (Sirkball Institute of Biomolecular Medicine, New York School of Medicine) and were grown in complete medium, consisting of Advanced DMEM (with nonessential amino acids, from Invitrogen), with 10% FBS, 10units/ml penicillin, and 10µg/ml streptomycin, 2mmol/L L-glutamine, and 55 µmol/L beta-mercaptoethanol. Full-length rat ATF-4 was cloned into pcDNA3.1 using the following primers: 

\[ \text{ATF-4 EcoRI FOR: (5’–GATACCAGCGAATTCTACAACATGACCG-3’)} \]  and \[ \text{ATF-4 XhoI REV: (5’–AGCACAAAGCCTCGAGTACCCATATA-3’)} \]. For transfection, cells were allowed to grow to 60-70% confluence, then transfected with indicated constructs using FuGENE6 (Roche Molecular Biochemicals).

Luciferase Assays

Firefly luciferase reporter constructs were transfected in growth quiescent rat aortic SMCs together with 1µg of the internal control plasmid pRL-TK (\textit{Renilla} luciferase driven by the thymidine
kinase promoter). Luciferase activity was measured 24h after transfection using the Dual Luciferase Assay System (Promega). Firefly luciferase activity was normalized with Renilla luciferase generated by pRL-TK to correct for transfection efficiency.

**Semi-quantitative RT-PCR**

Total RNA was prepared from cells that were injured or exposed to FGF-2 with TRIzol in accordance with the manufacturer’s instructions (Life Technologies, Inc.). RNA was reverse-transcribed to cDNA using oligo(dT) primers and Superscript II (Life Technologies). PCR was performed in 20µL containing 2.5mmol/L MgCl₂, 0.1mmol/L dNTP, 0.1µmol/L primers, 1µL cDNA, and 1U Platinum Taq Polymerase (Invitrogen) using an Applied Biosystem Gene-Amp PCR system 2400 (Perkin Elmer). For ATF-4 PCR, cycling conditions were 94°C for 30 s, 22 cycles of 95°C for 10 s; 61°C for 30 s, and 72°C for 1min, and an extension at 72°C for 4min. Similar conditions were used for other genes, with the following variations in annealing temperature and cycle number: for VEGF-A 61°C, 30 cycles; for Egr-1 60°C, 32 cycles; for PDGF-A, 57°C, 25 cycles; for Ch25h 60 °C, 30 cycles; for PDGF-Ralpha 60°C, 20 cycles; for ATR2 60°C, 35 cycles; and for GAPDH 58°C, 21 cycles. Primer sequences for ATF-4 were ATF-4 For (5’-CCTCGATACCAGCAAATCCCTACAAC-3’) and ATF-4 Rev (5’-GAGATATCAACCTCAGTTCCCCGCTCAAC-3’); for VEGF-A were VEGF-A For (5’-GCTCTCTGTTGGAAGACTGGA-3’) and VEGF-A Rev (5’-CCTTGGAACAGCGGATGTGTGT-3’); for Egr-1 were Egr-1 For (5’-CTTACTCCTCTGGGCTCTCTACAAC-3’) and Egr-1 Rev (5’-CCCTCCCTTTGCTCTTTTACCTAC-3’); for PDGF-A were ratPDGFAf97 5’-CCTCCCTTTTGTCTCTTCCCTAC-3’ and ratPDGFAr710 (5’-CCGTCCTCTCCTCCCGTGATGTGTC-3’); for Ch25h were Ch25h For (5’-TGAGTCACGGTCTCTTGAC-3’) and Ch25h Rev (5’-GGAGTCCAGCTCAGCTCAC-3’); for PDGF-Ralpha were PDGF-Ralpha For (5’-
TCGAGTGGATGATCTGCAAG-3') and PDGF-Ralpha Rev (5'-
TCCCATCTGGAGTCGTAAGG-3'); for ATR2 were ATR2 For (5'-
GAATCCCTGGCAAGCATCTTATGTA-3') and ATR2 Rev (5'-
CAGAGGTGTCCATTTCTCTAACAGA-3'), and for GAPDH, GAPDH For (5'-
ACCACAGTCCATGCCATCAC-3') and GAPDH Rev (5'-TCCACCACCCTGTTGCTGTA-3').

Linearity of gene expression was established by cycle-based RT-PCR in pilot experiments for
ATF-4, VEGF-A, Egr-1, PDGF-A, Ch25h, PDGF-Ralpha, ATR2 and GAPDH.

Quantitative Real Time RT-PCR (QRT-PCR) Assays

The QRT-PCR was carried out using SYBR Green PCR Master Mix (Applied Biosystems).
Briefly, total RNA was isolated and reverse-transcribed as described above. PCR was performed
in a 10µL reaction, consisting of 5µL SYBR Green PCR Master Mix, and 0.3µmol/L of forward
and reverse primers. PCR amplification was done using a Corbett Rotor Gene RG-3000 system.
The PCR conditions consisted of an initial hold step of 50°C for 2min, followed by activation at
95°C for 10min, followed by 40 cycles of 95°C for 10 s, 60°C for 15 s and 72°C for 10 s. The Ct
(threshold cycle) value of ATF-4 or VEGF-A 164 amplification was normalized to that of
GAPDH control. PCR products were visualised on EtBr-stained agarose gels to ensure product of
right size and no primer dimerization. All runs were performed with simultaneous generation of
standard curves (Reaction efficiencies = 1.10 10^(-1/m)–1, and R values = 0.99, for ATF-4,
VEGF-A, and GAPDH, data not shown). The primers for QRT-PCR were the following: for
GAPDH, Rat GAPDH For (5'-ACAAGATGGTGTAAGGTCGGTG-3’) and Rat GAPDH Rev (5'-
AGAAGGCAGCCCTGGTAACC–3’; for ATF-4, ATF-4 For (5’-CCTCGATACCAAGCAAA
TCCCTACAAAC-3’) and ATF-4 Real Rev (5’–GTCATCTAAGAGACCTAGGCTT–3’); for
VEGF-A, *VEGF-A Real For* (5′-TCACCAAGCCAGCACATAG-3′) and *VEGF164 Real Rev* (5′–GCTCACAGTGATTTTCTG GC–3′).

**Small Interfering RNA**

Rat SMCs were seeded in Petri dishes and growth arrested with serum-free medium 6h before transfection with 0.4 μmol/L small interfering RNA (siRNA; Qiagen) targeting endogenous rat ATF-4. Twenty-four h after serum-arrest, the cells were incubated with FGF-2 as indicated. Total RNA was extracted using the TRIzol method; cDNA synthesis and QRT-PCR were performed. ATF-4 siRNA targeting nucleotides 615-636 (GenBank Accession No. BC061546) was: 5′-r(GCCμGACUCμGμGCUUAU)d(TT)-3′ and its corresponding complementary strand 5′-r(AUAAGCAGCAGAGUCAGGC)d(TT)-3′. ATF-4 siRNAscr: 5′-r(μGUCACUUAGAAUCUCCG)d(TT) -3′/5′-r(GCGGAGAUUCUAAGμGACA)d(TT)-3′.

**Immunoprecipitation and Western Blot Analysis**

Lysates of cells exposed to FGF-2 were pre-cleared with equal volumes of Protein G- and A-Sepharose 4 Fast Flow Beads (Amersham) for 1h at 4°C before addition of the ATF-4 antibodies (sc-22800, Santa Cruz) or NF-kappaB p65 antibodies (sc-109, Santa Cruz), and gentle shaking overnight at 4°C. Pull-downs were performed using fresh beads during 24h with shaking. After sequential washing, SDS loading buffer was added to the beads, boiled, and loaded onto denaturing 10% SDS-polyacrylamide gel and resolved for 2h at 100 V. After transfer of proteins to Immobilon P nylon membranes (Millipore, Bedford, MA) and blocking non-specific binding sites with non-fat skim milk, membranes were incubated with ATF-4 antibody (1:1000) prior to chemiluminescence detection (PerkinElmer Life Sciences). To check equal loading, equivalent volumes of cell lysates per sample were resolved on SDS-PAGE, transferred to nylon membranes,
and probed with Sp1 (sc-420, Santa Cruz Biotechnology). For analysis of expression of the expression of ER stress markers in SMCs, p-eIF2 alpha (Ser51) (9721, Cell Signalling) and eIF2 alpha (9722, Cell Signalling) were used in Western blots of FGF-2-treated and injured rat SMC whole cell lysates.

**Electrophoretic Mobility Shift Analysis**

Nuclear extracts of FGF-2 treated cells were prepared as described previously. Binding reactions were performed using 10 µg of nuclear extract in 20µL containing 1µg of poly(dI-dC)-poly(dI-dC) (Sigma), 12.5mmol/L HEPES, pH7.9, 10% glycerol, 100mmol/L KCl, 1mmol/L EDTA, 1mmol/L dithiothreitol, 5µg acetylated BSA, and the 32P-labeled oligonucleotide for 20min at 25°C. In competition experiments, 100x molar excess of unlabeled oligonucleotide was included in the mixture 20min prior to addition of the probe. Bound complexes were separated from the unbound probe by nondenaturing 6.5% polyacrylamide gel electrophoresis in 1xTBE running buffer at 100V. The gels were vacuum-dried and exposed to Hyperfilm-MP (Amersham Pharmacia Biotech) overnight at -80°C. Oligonucleotides were: Oligo VEGF-A1752/1786 5’-GCGCGAGCCGATTACATCAGCCCGGGCCT-3’, putative ATF-4 binding site underlined; mOligo VEGF-A 1752/1786 5’-GCGCGAGCCAATCATACAAAGCCCGGGCCT-3’; ATF-4 Oligo 5’-CTCGAGGGCTGGGCGGCGGTACCAGTGACGTGAGTTGCGGAGGAG-3’. Human recombinant ATF-4 protein (Abnova H00000468-P01) was used as a positive control.

**ChIP**

Human aortic SMCs grown in 100-mm dishes were serum-arrested for 24h, and treated with FGF-2 for 2h. The samples were processed as previously described. PCR was performed in a reaction consisting of 2.5mM MgCl2, 0.1mM dNTP, 0.1µM primers and 1U Platinum Taq polymerase
Cycling conditions were as follows: 98°C for 1min; 40 cycles of 98°C for 30s; 58°C for 30s and 72°C for 30s, with a 2min extension time. Human VEGF-A promoter was amplified using primers VEGF_For137516: 5’-TGCCACTCGGTCTCTTCAGC-3’ and VEGF_Rev137743: 5’-CCGAGGCTCCGGAACAC-3’.

**Signaling Inhibitors**

Established inhibitors of two known pathways were used 1h prior to 4h FGF-treatment: LY294002 (10µmol/L) for PI3K, and PD98059 (10µmol/L) for ERK. All inhibitors were obtained from Calbiochem. Cells were then exposed to FGF-2 for 4h, then total RNA was isolated for QRT-PCR analysis.

**SMC Proliferation**

Growth quiescent SMCs in 96 wps were incubated with VEGF-A or FGF-2 and cell numbers were determined after 72h using an automated Coulter counter. Alternatively the cells were transfected with pcDNA3-ATF-4 (in FuGENE6), incubated with neutralizing antibodies to VEGF-A (AF564, R&D Systems) or IgG for 72h, and then counted.

**Rat Carotid Artery Injury Model**

Balloon catheter injury to the carotid artery of adult Sprague Dawley rats (450-550g) was performed essentially as previously described prior to sacrifice 4h, 5d and 14d after injury. The rats were obtained from the Baker Medical Research Institute, Melbourne, Australia. Left common carotid arteries were subjected to balloon catheter injury by surgical procedures approved by the Baker Medical Research Institute and Alfred Hospital Animal Experimentation Committee. Tissue processing and morphometry were performed on elastin-stained cross-sections, in a blinded
manner. Cross-sectional areas of the media, the neointima and overall vessel size were measured using a computer-interfaced imaging system (Optimus Bioscan2, Thomas Optical Measurement system, Inc). 4-5 rats were used in each group. Where indicated, 100µl of PBS, pH7.4, containing 10µl FuGENE6 and 50 µg siRNA, siRNAscr or the vehicle was infused into the ligated segment for 20min at 100mm Hg.

**Immunohistochemical Detection of ATF-4 and VEGF-A in Rat Carotid Arteries**

Immunohistochemical analysis was performed with antibodies to ATF-4 (sc-22800, Santa Cruz; 1:200), VEGF-A (sc-152, Santa Cruz, 1:200), Akt (pan)(11E7)(4685, Cell Signalling, 1:300) and p-Akt (Ser473) (3787S, Cell Signalling, 1:300) on consecutive paraffin sections of formalin-fixed balloon-injured rat carotid arteries as described previously². Primary antibodies were omitted as negative controls.

**Replicates and Statistics**

For data presented in histograms, results were expressed as mean values ± SD from tri- or quadruplicate measurements performed in 2 to 4 independent experiments producing similar results. For gel-based display items, the data is representative of two or more independent experiments. Data was analysed for statistical significance (P<0.05) using two-tailed Student’s t-test or analysis of variance (ANOVA) between groups.

**Reference**