Vascular smooth muscle cell (VSMC) proliferation and migration contribute significantly to atherosclerosis, postangioplasty restenosis, and transplant vasculopathy. Forkhead transcription factors belonging to the FoxO subfamily have been shown to inhibit growth and cell cycle progression in a variety of cell types. We hypothesized that forkhead proteins may play a role in VSMC biology. Under *in vitro* conditions, platelet-derived growth factor (PDGF)-BB, tumor necrosis factor-α, and insulin-like growth factor 1 stimulated phosphorylation of FoxO in human coronary artery smooth muscle cells via MEK1/2 and/or phosphatidylinositol 3-kinase-dependent signaling pathways. PDGF-BB, tumor necrosis factor-α, and insulin-like growth factor 1 alone down-regulated the FoxO target gene, p27kip1, and enhanced cell survival and progression through the cell cycle. These effects were abrogated by overexpression of a constitutively active, phosphorylation-resistant mutant of the FoxO family member, TM-FKHR1. The anti-proliferative effect of TM-FKHR1 was partially reversed by small interfering RNA against p27kip1. In a rat balloon carotid arterial injury model, adenovirus-mediated gene transfer of FKHR1 caused an increase in the expression of p27kip1 in the VSMC and inhibition of neointimal hyperplasia. These data suggest that FoxO activity inhibits VSMC proliferation and activation and that this signaling axis may represent a therapeutic target in vasculopathic disease states.

Atherosclerosis, a leading cause of mortality and morbidity in the Western world, involves a multitude of pathophysiological processes including endothelial dysfunction, inflammation, vascular smooth muscle cell (VSMC) proliferation, and activation in the extracellular matrix (1, 2). Percutaneous transluminal coronary angioplasty is a well established procedure for revascularization of patients with arterial occlusive disease. The occurrence of restenosis at the site of angioplasty remains a major limitation of this procedure. An important goal is to elucidate the molecular basis of restenosis and to use this information to identify novel therapeutic targets.

Neointimal hyperplasia, a hallmark of VSMC proliferation, contributes significantly to the restenotic process after percutaneous transluminal coronary angioplasty. The complex response involves initial accumulation of inflammatory cells and the release of growth factors and chemotactic cytokines (3). Various studies have implicated a role for platelet-derived growth factor (PDGF), tumor necrosis factor (TNF)-α, and insulin-like growth factor (IGF)-1 in this process (4–15).

PDGF-BB, which is expressed in multiple cell types, including VSMC, is a potent mitogen for VSMC in *vitro* and in *vivo* (16–21). PDGF has been shown to be secreted from platelets at sites of intimal injury and/or thrombus (22, 23). Several recent studies have reported that inhibition of PDGF or PDGF receptor attenuates neointimal hyperplasia (24–26). Others demonstrated a direct association between PDGF expression and neointimal thickening (27–29).

TNF-α is a pluripotent mediator of inflammation (30) and is believed to play a pathophysiological role in vascular remodeling. TNF-α promotes migration of cultured VSMC (31, 32) and induces the expression of growth factors and cytokines (33), adhesion molecules (34), and extracellular matrix-degrading metalloproteinase (35). Under *in vivo* conditions, TNF-α is expressed by macrophages and VSMC in atherosclerotic plaques but not in normal vessels (36–38). In various animal models, TNF-α levels are increased following balloon injury, following wire carotid injury, following common carotid artery ligation, and during acute rejection of cardiac allografts (39, 39–42). Blockade of TNF-α with a soluble TNF-α receptor inhibits acute coronary neointimal formation in an animal model of coronary graft atherosclerosis (43). Finally, injury- or ligation-induced intimal hyperplasia of the carotid artery is markedly attenuated in mice that are null for TNF-α (40, 42, 44).

IGF-1 is a weak mitogen for VSMC in *vitro*. Previous studies have demonstrated increased levels of IGF-1 in the region of intimal hyperplasia in rabbit aorta (45) and in the neointima of RNA; CASMC, coronary artery smooth muscle cell(s); SmBM, smooth muscle basal medium; FBS, fetal bovine serum; Adv, adenovirus encoding the cDNA for β-galactosidase; WT-FKHR1, wild type FKHR1; TM-FKHR1, triple mutant FKHR1; DAPI, 4′,6-diamidino-2-phenylindole; FACS, fluorescence-activated cell sorting; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PI3K, phosphatidylinositol 3-kinase.
arteriovenous fistulas (46). In addition, IGF-1 receptor is highly expressed in the VSMC of intact arteries and in cultured VSMC (7, 47).

The forkhead winged helix transcription factor, DAF-16, was shown to regulate longevity and lipid metabolism in Caenorhabditis elegans (48). Mammalian homologues of DAF-16 (namely members of the FoxO family (FKHR, FKHRL1, and AFX)) have been implicated in cell growth, cell cycle, and differentiation (49, 50). In various cell types, insulin and IGF-1 induce phosphorylation of FoxO proteins, which results in their exclusion from the nucleus and decreased expression of downstream target genes involved in programmed cell death and cell cycle arrest (51, 52). Recently, we demonstrated a role for forkhead proteins in mediating endothelial cell cycle arrest and apoptosis (53, 54). In the current study, we show that FoxO proteins lie downstream of PDGF-BB, TNF-α, and IGF-1 signaling pathways in VSMC in vitro, and we provide evidence for an inhibitory role of forkhead proteins in injury-induced neointimal hyperplasia in vivo.

EXPERIMENTAL PROCEDURES

Materials—PD98059, LY294002, and wortmannin were purchased from Calbiochem. Recombinant human PDGF-BB, TNF-α, and IGF-1 were purchased from R&D Systems (Minneapolis, MN). Antibodies to AKT, phospho-AKT-Ser-473, FKHR, FKHRL1, and FKHR-Ser-256 (which also recognizes phospho-AFX-Ser-193), β-actin, and hemagglutinin (HA) were from Cell Signaling (Beverly, MA). Cy3-conjugated secondary antibodies included anti-rabbit IgGs, streptavidin Texas Red and fluorescein isothiocyanate or anti-mouse IgG/horseradish peroxidase conjugate (1:1000 dilution; Amersham Biosciences). RNA was harvested with the Trizol reagent, and hybridization was performed with p21vds CDNA probe as previously described (53). Northern and Western blots were performed in triplicate, and representative blots are shown.

Adenovirus Vector Constructs and Transduction—CASM C were transduced with replication-defective adenovirus encoding the cDNA for I-galactosidase (Adv), wild-type FKHRL1 (WT-FKHRL1), and the phosphorylation-resistant triple mutant FKHRL1 (TM-FKHRL1), which contains T32A, S253A, and S319A (53). For virus transductions, CASMC were plated at ~70–80% confluence and cultured for 5 h at 37 °C, 5% CO2 in smooth muscle growth medium (SmGM, Cambrex Bio Science). The cells were washed once with and incubated in Opti-MEM I (without phenol red) for 30 min and then transduced at a multiplicity of infection of 20 in SmGM medium for 12 h, grown in fresh medium for another 10 h, and then serum-starved in SmBM for 16–72 h as indicated.

FKHR and FKHRL1 Immunofluorescence—CASM C were plated onto 4-well chamber slides (Lab-Tek, Christchurch, New Zealand) at a density of 50,000 cells/well. The cells were fixed in 3.7% paraformaldehyde for 20 min at room temperature, washed with phosphate-buffered saline (PBS) and incubated in blocking buffer (4% normal goat serum, 0.1% saponin in phosphate-buffered saline) for 10 min. The cells were incubated with anti-FKHR, anti-FKHRL1, or anti-HA antibody (1:100) in 200 μl of blocking buffer for 1 h at room temperature, followed by fluorescein isothiocyanate-conjugated secondary anti-rabbit IgG (1:100) in 200 μl of blocking buffer for 1 h. After extensive washing, the cells were incubated with DAPI-containing mounting medium Vectashield (Vector Laboratories, Burlingame, CA) for 10 min at room temperature, and representative images were captured with a Nikon Eclipse E800 microscope and a Spot digital camera. The images were merged using Adobe Photoshop software.

Proliferation Assays—CASM C were grown to a density of 50,000 cells/well in 12-well plates. At 80% confluence, the cells were starved for 48 h in SmBM in the absence of serum. The cells were treated with TNF-α, PDGF-BB, or 10% fetal calf serum for 2 h to induce cell cycle reentry and pulsed with 1 μCi/liter [3H]thymidine (Amersham Biosciences) during the last 4 h of incubation. After washes with cold phosphate-buffered saline, cells were trypsinized and solubilized with 0.2 N NaOH. Radioactivity incorporated into DNA was measured in a scintillation counter. In addition, the XTT assay was employed to determine cell proliferation. This assay is based on the conversion of the yellow tetrazolium salt XTT into an orange, water-soluble dye, formazan, by metabolically active cells. Cells were serum-starved for 48 h, treated with agonists for 48 h, and then incubated with XTT for 4 h. Formation of formazan was directly quantitated in 96-well plates with an enzyme-linked immunosorbent assay reader at A490–655 nm (model 680; Bio-Rad). Trypan blue exclusion method was also used to count the number of viable cells where indicated (55).

The membrane was blocked with TBST (10 mM Tris, 0.2% Tween 20) containing 5% nonfat milk for 1 h and then incubated with primary antibody for 2 h at room temperature. After three washes with TBST, the membrane was incubated with secondary antibody for 1 h at room temperature. The enhanced chemiluminescence kit (Pierce) was used for detection. Membranes were stripped with Restore Western blot stripping buffer (Pierce) for 15 min at room temperature followed by washing with TBST. Primary antibodies were used as indicated. Secondary antibodies included anti-rabbit IgG/IgG/horseradish peroxidase conjugate or anti-mouse IgG/horseradish peroxidase conjugate (1:1000 dilution; Amersham Biosciences). RNA was harvested with the Trizol reagent, and hybridization was performed with p21vds CDNA probe as previously described (53). Northern and Western blots were performed in triplicate, and representative blots are shown.

**Fig. 1.** PDGF-BB, TNF-α, and IGF-1 each induces phosphorylation of forkhead proteins in CASMC. CASMC were grown to 85–90% confluence, serum-starved for 16 h in basal SmBM medium, and then treated with PDGF-BB (10 ng/ml for 15 min) (A), TNF-α (10 ng/ml for 30 min) (B), or IGF-1 (100 ng/ml for 15 min) (C) and processed for Western blot analyses of phospho-FKHR-Ser-256, phospho-AFX-Ser-193, and phospho-AKT-Ser-473. Membranes were stripped and reprobed for total Akt and β-actin as loading controls. Where indicated, serum-starved cells were pretreated with 50 μM MEK1/2 inhibitor, 50 μM PD98059 (PD), or 50 μM of PI3K inhibitor, LY294002 (LY), for 30 min. The blots shown are representative of three independent experiments.
FIG. 2. PDGF-BB, TNF-α, and IGF-1 each induces nuclear exclusion of forkhead proteins in CASMC. A total of 50,000 cells of CASMC were cultured in 4-well chamber slides; serum-starved for 24 h; treated in the absence or presence of PDGF-BB, TNF-α, and IGF-1 for 30 min; fixed with paraformaldehyde; and then incubated with primary antibodies to FKHR (A) or FKHRL1 (B), followed by Cy3-conjugated anti-IgG secondary antibody. The cells were incubated with DAPI for nuclear staining and observed under fluorescence microscope. Quantitation of the distribution of FKHR and FKHRL1 in the nucleus (N) and cytoplasm (C) of CASMC treated with or without the growth factors/cytokines indicated is shown in the bottom panel of A and B, respectively. Mean ± S.D. from 100 cells are shown. C, quantification of nuclear-cytoplasmic distribution of FKHR in CASMC treated with or without (control) PDGF-BB, TNF-α, or IGF-1 and pretreated in the absence or presence of the MEK1/2 and PI3K inhibitors (PD98059 and LY294002, respectively). Mean ± S.D. from 100 cells are shown. N, nucleus; C, cytoplasm. Immunofluorescence experiments were carried out in triplicate.
Flow Cytometry for Cell Cycle and Apoptosis Assay—FACS analyses were carried out to determine apoptosis as assayed by annexin V-propidium iodide staining of intact cells and to determine the cell cycle distribution of CASMC by quantifying propidium iodide-labeled DNA content of ethanol-permeabilized cells, as previously described (53). A total of 10,000 cells were counted (gated) by FACS.

In Vitro Knockdown of p27kip1 by siRNA—p27kip1 siRNA was synthesized using the DNA target sequence: AAGTAGGTAAGCCGCAAGAGGTG (56). 400,000 CASMC were plated onto a 60-mm plate, and 24 h later, the cells were transfected with 5 μg of siRNA p27kip1. In brief, 25 μl of Lipofectin was added to 225 μl of Opti-MEM I in a polystyrene tube. After a 30-min incubation at room temperature, the mixture was added to the cells. The cells were washed with Opti-MEM I and then incubated with serum-free medium for 16 h and then treated without or with PDGF-BB for 4 h were processed for Western blot analyses of p27kip1 protein. Membranes were stripped and reprobed for β-actin as loading control. Data are given as mean ± S.E. of at least three independent experiments. The cells that were processed for an additional 30 min. 2 ml of fresh Opti-MEM I was added to each polystyrene tube containing 500 μl of the mixture of siRNA and Lipofectin. The cells were washed with Opti-MEM I and then incubated with 2.5 ml of the above mixture at 37 °C, 5% CO2. After 4.5 h, Opti-MEM I was removed, and cells were incubated in SmGM medium for 24–48 h, as indicated. For co-transduction with adenovirus and siRNA, the cells were plated and transduced with the viruses expressing either control β-galactosidase (Adv) or TM-FKHRL1 on day 1 and then transfected with either NS or p27kip1 siRNA on the next day.

Gene Transfer and in Vivo Balloon Angioplasty—The rat carotid balloon angioplasty model was performed according to the method described by Clowes et al. (57, 58). Briefly, 4-month-old Sprague-Dawley rats (350–400 g) were heparinized systemically (100 units/kg). 1–1.5 cm of the left carotid arteries of adult males were injured with a 2F embolectomy catheter (Baxter Edwards Healthcare, Irvine, CA), which was inflated in the carotid and passed three times to achieve significant injury. The vessel was filled with 50 μl of normal saline (n = 8) or with 105 to 106 plaque-forming units of rAd.TM-FKHRL1 (n = 14) or control rAd.β-gal (n = 6) for 20 min. The adenovirus solution was flushed out via the opened external carotid artery. The external carotid was then ligated, and blood flow was restored. All rats were housed at the BIDMC animal facility and were treated according to published National Institutes of Health guidelines.

Tissue Collection, Morphology, and Immunocytochemistry—Rats subjected to balloon injury were monitored for 14 days and subsequently sacrificed. Injured carotids were retrieved, fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin/eosin for histomorphometric analysis. The ratio of intima versus media (IM) in each carotid was measured by morphometric analysis using the NIH Image version 1.62 public domain software. IM ratios from 10 serial sections 50 μm apart were averaged for each animal. To detect PDGF-B in the tissue sections, immunochemical staining was performed using biotinylated anti-IgG as a secondary antibody. Apoptotic cells were identified using the Vectastain Elite ABC kit. Immunofluorescent staining of 5-μm frozen sections was employed to detect Ki-67, smooth muscle actin, HA, FKHRL1, and p27kip1. The snap frozen tissue sections were fixed with 2% paraformaldehyde followed by the heat treatment in 10 mm citrate buffer.

Statistical Analysis—Data are given as mean ± S.E. of at least three independent experiments. Statistical analysis was performed by analysis of variance as appropriate.

RESULTS

PDGF-BB, TNF-α, and IGF-1 Induce Phosphorylation of FoxO Proteins in CASMC—To determine whether PDGF-BB, TNF-α, and IGF-1 modulate the phosphorylation of forkhead proteins in VSMC, we carried out Western blot analyses of CASMC treated in the absence or presence of the cytokine/growth factor. As shown in Fig. 1, incubation of CASMC with PDGF-BB (10 ng/ml for 15 min), TNF-α (10 ng/ml for 30 min), or IGF-1 (100 ng/ml for 15 min) each resulted in phosphorylation of Akt at serine 473, FKHR at serine 256, and AFX at serine 193. PDGF-BB-mediated induction of FKHR phosphorylation was blocked by pretreatment with the PI3K inhibitors, LY294002 and wortmannin, but not by the MEK1/2 inhibitor,
PD980059 (Fig. 1A shows LY294002 and PD980059). In contrast, PDGF-BB-induced phosphorylation of AFX was only partially inhibited by LY294002. The effect of TNF-H9251 on the phosphorylation of FKHR and AFX was partially attenuated by LY294002 and PD980059 (Fig. 1B). Finally, IGF-1-mediated phosphorylation of FKHR and AFX was blocked by LY294002 (Fig. 1C). In time course experiments, the effect of TNF-H9251 on phosphorylation of FKHR and AFX was delayed (maximum at 30 min) compared with PDGF-BB and IGF-1 (maximum at 15 min) (data not shown). Taken together, these data indicate that PDGF-BB, TNF-H9251, and IGF-1 each induces phosphorylation of forkhead in CASMC through MEK1/2 and/or PI3K-dependent signaling pathways.

PDGF-BB, TNF-H9251, and IGF-1 Induce Nuclear Export of FKHR and FKHRL1 in CASMC—

To determine the effect of PDGF-BB, TNF-α, or IGF-1 on nuclear localization of FoxO, CASMC were cultured in 4-well chamber slides, serum-starved as described under "Experimental Procedures" and PD980059 (Fig. 1A shows LY294002 and PD980059). In contrast, PDGF-BB-induced phosphorylation of AFX was only partially inhibited by LY294002. The effect of TNF-α on the phosphorylation of FKHR and AFX was partially attenuated by LY294002 and PD980059 (Fig. 1B). Finally, IGF-1-mediated phosphorylation of FKHR and AFX was blocked by LY294002 (Fig. 1C). In time course experiments, the effect of TNF-α on phosphorylation of FKHR and AFX was delayed (maximum at 30 min) compared with PDGF-BB and IGF-1 (maximum at 15 min) (data not shown). Taken together, these data indicate that PDGF-BB, TNF-α, and IGF-1 each induces phosphorylation of forkhead in CASMC through MEK1/2 and/or PI3K-dependent signaling pathways.

PDGF-BB, TNF-α, and IGF-1 Induce Nuclear Export of FKHR and FKHRL1 in CASMC—

To determine the effect of PDGF-BB, TNF-α, or IGF-1 on nuclear localization of FoxO, CASMC were cultured in 4-well chamber slides, serum-starved for 24 h, and then treated with or without 10 ng/ml PDGF-BB, 10 ng/ml TNF-α, or 100 ng/ml IGF-1 for 30 min. Subcellular distribution of FKHR and FKHRL1 was assayed by immuno-fluorescence. Under serum starvation conditions, FKHR and FKHRL1 were predominantly localized in the nucleus. Treatment with PDGF-BB, TNF-α, or IGF-1 resulted in nuclear export of FKHR and FKHRL1 (Figs. 2, A and B). Preincubation
of CASMC with 50 μM LY294002 significantly inhibited PDGF-BB-, TNF-α, and IGF-1-mediated nuclear exclusion of FKHR, whereas the inhibitory effect of 50 μM PD98059 was limited to TNF-α-mediated translocation of FKHR (Fig. 2C). These results are consistent with the phosphorylation data (see Fig. 1) and collectively suggest that PDGF-BB-, TNF-α-, and IGF-1-mediated phosphorylation of FoxO results in cytoplasmic translocation/nuclear exclusion of the transcription factors.

PDGF-BB Suppresses p27kip1 Gene Expression in CASMC by a Forkhead-dependent Pathway—Previous studies in non-VSMC cells have demonstrated a link between forkhead activity and expression of the cell cycle inhibitor/apoptotic gene, p27kip1 (53, 59–64). Since PDGF-BB, TNF-α, and IGF-1 each phosphorylated FKHR and AFX and resulted in nuclear exclusion of FKHR and FKHRL1 (see Figs. 1 and 2), we examined the effects of these cytokines/growth factors on forkhead-mediated target gene expression in CASMC. Fig. 3A shows that PDGF-BB significantly inhibited endogenous p27kip1 mRNA expression. TNF-α resulted in a slight increase in p27kip1 transcript levels, whereas IGF-1 had little or no effect. As expected, adenovirus-mediated overexpression of TM-FKHRL1 increased basal levels of p27kip1 mRNA in CASMC (Fig. 3B). The constitutively active TM-FKHRL1 increased the trypan blue exclusion method (data not shown). In FACS analyses, PDGF-BB was shown to promote cell cycle progression into S phase, with a corresponding decrease in the number of cells in G1/G0 phase and an increase in the cells traversing G2/M phase (Fig. 6A). These effects were completely abrogated by TM-FKHRL1 but not by WT-FKHRL1 (Fig. 5B). The proliferative effect of 10% FBS was only partially inhibited (by 50%) by overexpression of TM-FKHRL1 (Fig. 5B). Similar results were obtained using the XTT proliferation assay (Fig. 5, C and D) and cell counting by the trypan blue exclusion method (data not shown). In FACS analyses, PDGF-BB was shown to promote cell cycle progression into S phase, with a corresponding decrease in the number of cells in G1/G0 phase and an increase in the cells traversing G2/M phase (Fig. 6A). These effects were completely abrogated by TM-FKHRL1 but not by WT-FKHRL1 (Fig. 6, B–D). Similar results were observed with FBS-treated cells. Neither TNF-α nor IGF-1 had a significant effect on VSMC cell cycle (Fig. 6A).

Taken together, these results indicate that forkhead protein plays a role in PDGF-BB and serum-mediated progression of the cell cycle.

Antiproliferative Effect of TM-FKHRL1 Is Partially Reversed by siRNA against p27kip1—Given that forkhead is coupled to the expression of p27kip1 in CASMC, we hypothesized that the inhibitory effects of TM-FKHRL1 on cell proliferation may be mediated, at least in part, by this cell cycle inhibitor. To test this hypothesis, CASMC were transduced with TM-FKHRL1 or control Adv, transduced with siRNA against p27kip1 or nonsilencing (NS), treated in the absence or presence of PDGF-BB, and then assayed for cell proliferation. Efficacy of siRNA against p27kip1 in CASMC was confirmed by Western
As shown in Fig. 7, TM-FKHR1-mediated inhibition of CASMC proliferation by PDGF-BB was indeed partially reversed by siRNA-mediated down-regulation of p27kip1. Taken together with our earlier findings, these data suggest that PDGF-BB-mediated proliferation of VSMC is dependent on nuclear exclusion of forkhead and secondary reduction of p27kip1 levels.

To confirm the above results in vivo, we studied a rat carotid balloon angioplasty model for neointimal hyperplasia. At 14 days after balloon injury, endogenous PDGF-BB was found to be highly expressed at the site of injury (Fig. 8). In the next set of experiments, we delivered adenovirus expressing β-galactosidase (rAd.β-gal) or TM-FKHR1 (rAd.FKHR1-TM) to balloon-injured carotid arteries of the rat. At day 14 following injury, significant neointimal hyperplasia was observed in the absence of adenovirus transduction (n = 8) or in the presence of rAd.β-gal (n = 6) with intima over media ratios (I/M) reaching 0.87 ± 0.33 and 0.92 ± 0.36, respectively (Fig. 9). Overexpression of rAd.FKHR1-TM (n = 14) resulted in a dose-dependent inhibition of neointimal hyperplasia with an I/M ratio of 0.35 ± 0.25 at the higher concentration (p < 0.01) (Fig. 9). In immunofluorescent studies of injured vessels at days 1 and 3, HA co-localized with smooth muscle cell antigen (SMA), confirming expression of exogenous HA-tagged TM-FKHR1 in VSMC (Fig. 10, A–D). Consistent with these results, TM-FKHR1-
transduced vessels demonstrated a marked increase in FKHRL1 immunostaining, compared with \( /H9252 \)-galactosidase control (Fig. 10, E–H). To gain insight into the mechanism of inhibition of neointimal hyperplasia by TM-FKHRL1, VSMC were assayed for proliferation and apoptosis \( \text{in vivo} \), using Ki-67 and the Vectastain Elite ABC kit, respectively. At day 3 following injury, Ki-67 expression was significantly reduced in HA-TM-FKHRL1-transduced arteries compared with the control (from 35 to 12% of DAPI-positive VSMC) (Fig. 10, I–L). In \( \text{in vivo} \) cell proliferation was detected by using anti-Ki-67 antibody (green) in the VSMC (red) of the \( /H9252 \)-galactosidase (M and O) and TM-FKHRL1-transduced (N and P) vessels at days 1 (M and N) and 3 (O and P) after injury. M–P, Vectastain Elite ABC kit was used to detect apoptotic cells (in blue) in VSMC and neointima at the site of injury in control (Q and S) and TM-FKHRL1-transduced (R and T) rat carotid arteries at day 1 and 3 as indicated. Q–T, \( p27^{kip1} \) (red) was detected in \( /H9252 \)-galactosidase-transduced (I and K) and TM-FKHRL1-transduced (J and L) vessels. Nuclei are shown in blue. Scale bars, 100 \( \mu \text{m} \) (A–D, I–P), 40 \( \mu \text{m} \) (E–H), and 15 \( \mu \text{m} \) (Q–T), as indicated.

**DISCUSSION**

Previous studies in other cell types have demonstrated a role for the forkhead family of transcription factors in mediating the effects of growth factors or serum on cell proliferation, cell cycle progression, and/or cell survival (49, 50, 53). When cells are exposed to growth factors or serum, the PI3K/Akt pathway is activated, resulting in the phosphorylation (at Thr-32, Ser-253, and Ser-315 in FKHRL1; at Thr-24, Ser-256, and Ser-319 in FKHR; and at Thr-28, Ser-193, and Ser-258 in AFX) and nuclear exclusion of forkhead transcription factors, down-regulation of target gene expression, and enhanced cell survival and proliferation. Putative forkhead-responsive genes include IGFBP-1, glucose-6-phosphatase, \( \text{FasL} \), TRAIL, GADD45A, BCL-6, BIM, and \( p27^{kip1} \). In contrast, the withdrawal of serum or growth factors leads to nuclear translocation of forkhead proteins and transcriptional activation of tar-
get genes, with subsequent cell cycle arrest and/or apoptosis.

The present study is the first to show that VSMC agonists, including PDGF-BB, TNF-α, and IGF-1, promote phosphorylation of FoxO proteins in VSMC, resulting in nuclear exclusion of the transcription factor. Moreover, the data suggest that forkhead activity is a critical determinant of VSMC proliferation both in vitro and in vivo.

PDGF-BB-mediated phosphorylation of forkhead proteins occurred primarily through PI3K, whereas the TNF-α and IGF-1 effect was dependent on both MEK1/2 and PI3K signaling. These findings are novel in that they provide the preliminary evidence for a PI3K-independent mechanism of forkhead regulation in VSMC. Whereas previous studies have implicated non-AKT kinases in forkhead signaling (61, 71–73), they have consistently demonstrated an absolute requirement for PI3K.

The forkhead-responsive gene, p27<sup>kip1</sup>, encodes a cyclin-inhibitory protein that plays an important role in regulating proliferation of many different cell types. Indeed, in a previous study, overexpression of p27<sup>kip1</sup> was reported to inhibit VSMC growth (74). Here, we show that whereas all three agonists (PDGF-BB, TNF-α, and IGF-1) induced phosphorylation and nuclear exclusion in VSMC, only PDGF-BB had a significant inhibitory effect on p27<sup>kip1</sup>. The reason for this discrepancy is not clear. Perhaps, PDGF-BB results in more intense and/or prolonged inactivation of FoxO in VSMC. Alternatively, TNF-α and IGF-1 may activate parallel signaling pathways that promote expression of p27<sup>kip1</sup>. Importantly, siRNA-mediated down-regulation of p27<sup>kip1</sup> partially reversed the ability of TM-FKHRL1 to block PDGF-BB mitogenicity. Together, these data suggest that PDGF-BB-mediated nuclear exclusion of forkhead and subsequent reduction of p27<sup>kip1</sup> expression is responsible, at least in part, for its growth-promoting activities.

In addition to p27<sup>kip1</sup>, another cell cycle inhibitor/anti-proliferative gene, BTG1, has been shown to be a target of the forkhead transcription factors in erythroid progenitors (75) and in human endothelial cells. We have found that PDGF-BB decreases expression of BFG1, like p27<sup>kip1</sup>, in CASMC in a forkhead-dependent manner, whereas IGF-1 and TNF-α had no such effect (data not shown). A role for BFG1 may explain why siRNA-mediated down-regulation of p27<sup>kip1</sup> only partially reversed the effect of TM-FKHRL1 on PDGF-BB mitogenicity.

The importance of p27<sup>kip1</sup> in mediating the inhibitory effects of FKHRL1 is further supported by our in vivo findings. In the carotid injury model, VSMC overexpressing FKHRL1 demonstrated significantly higher levels of p27<sup>kip1</sup> expression and lower levels of proliferation, compared with β-galactosidase (Adv) control. Consistent with these results, Braun-Dallaeus et al. (76) demonstrated that p27<sup>kip1</sup> is expressed at high levels in uninjured arteries and is rapidly down-regulated after balloon injury. Overexpression of p27<sup>kip1</sup> in cultured VSMC resulted in G<sub>1</sub> arrest and complete inhibition of proliferative response to serum, whereas adenoviral transfer of p27<sup>kip1</sup> in a rabbit model of balloon injury resulted in significant inhibition of intimal cell proliferation (77). However, our in vivo data do not exclude a role for other forkhead target genes (e.g. BFG1) in mediating the inhibitory effects of TM-FKHRL1 on neointimal hyperplasia.

Following the original submission of this manuscript, a study was published describing that constitutively active TM-FKHRL1 induces p27<sup>kip1</sup> protein in rat aortic vascular smooth muscle cells and that overexpression of TM-FKHRL1 leads to cell cycle arrest and apoptosis (78). Balloon injury resulted in rapid down-regulation of phospho-FKHRL1 and a delayed reduction in p27<sup>kip1</sup> in the blood vessel wall. Finally, overexpression of TM-FKHRL1 resulted in a significant increase in p27<sup>kip1</sup>

expression, lower proliferative index, increased apoptosis, and reduction in neointimal area (78).

The findings of our present study add to the latter report in several important ways. First, we have shown that human coronary artery vascular smooth muscle cells express not only FKHRL1, but also FKHR and AFX. Second, we have demonstrated that growth factors induce phosphorylation and nuclear exclusion of endogenous forkhead proteins in VSMC. Third, we have delineated the signaling pathways involved in agonist-mediated phosphorylation of endogenous forkhead and consequent down-regulation of the forkhead target gene, p27<sup>kip1</sup>, at the transcript and protein levels. Fourth, we have employed an siRNA approach to provide direct evidence for the role of p27<sup>kip1</sup> in the PDGF-BB-forkhead-cell proliferation pathway. Finally, our in vivo results contrast with those of the previous study in that they fail to demonstrate TM-FKHRL1-mediated changes in VSMC apoptosis, using two different assays. It is conceivable that TM-FKHRL1 induces cell cycle arrest in vitro, without promoting apoptosis. Alternatively, apoptotic cells may be promptly removed from the site of injury.

There is increasing evidence that bone marrow-derived progenitors contribute to neointimal formation in response to vascular injury (79–83). An interesting question is whether progenitor-derived VSMC are more or less prone to the inhibitory effects of forkhead overexpression, compared with locally derived VSMC. Moreover, it is interesting to speculate whether forkhead-mediated changes in gene transcription in the native vessel wall results in reduced homing of circulating progenitor cells to the injury site.

In conclusion, we have shown that growth factor and cytokine signaling is linked to forkhead transcription factor(s) in VSMC. Our data suggest that forkhead signaling pathway contributes to the regulation of VSMC proliferation, cell cycle progression, and neointimal hyperplasia. Thus, the forkhead pathway may represent a novel therapeutic target in vasculopathic diseases.

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Footnotes:

2 M. R. Abid and W. C. Aird, unpublished data.
