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Objective—We examined the effects of FKHRL1 (forkhead transcription factor in rhabdomyosarcoma like-1) overexpression on vascular smooth muscle cell (VSMC) proliferation, apoptosis, and cell cycle, in vitro, and the role of FKHRL1 and p27 in the pathophysiology of neointimal growth after balloon angioplasty, in vivo. Furthermore, we tested whether FKHRL1 overexpression can inhibit neointimal hyperplasia in a rat carotid artery model.

Methods and Results—Adenovirus expressing the constitutively active FKHRL1 (FKHRL1-TM; triple mutant) with 3 Akt phosphorylation sites mutated was transfected to subconfluent VSMCs. FKHRL1 overexpression in cultured VSMCs increased p27 expression, leading to G1 phase cell-cycle arrest and increased apoptosis. In vivo, the phosphorylation of FKHRL1 increased significantly 3 hours after balloon injury and decreased thereafter, with the subsequent downregulation of p27. Although the phosphorylation of FKHRL1 was greatest at 3 hours, the downregulation of p27 showed a temporal delay, only slightly starting to decrease after 3 hours and reaching a nadir at 72 hours after balloon injury. Gene transfer of FKHRL1-TM increased p27, decreased proliferation, and increased apoptosis of VSMCs, which resulted in a marked reduction in neointima formation (intima-to-media ratio: 0.31 ± 0.13 versus 1.17 ± 0.28, for FKHRL1-TM versus Adv-GFP; $P<0.001$).

Conclusion—Balloon angioplasty leads to the phosphorylation of FKHRL1 and decreased expression of p27, thereby promoting a proliferative phenotype in VSMCs in vitro and in vivo. This study reveals the importance of FKHRL1 in proliferation and viability of VSMCs and suggests that it may serve as a molecular target for interventions to reduce neointima formation after angioplasty. (***Arterioscler Thromb Vasc Biol*. 2005;25:742-747.)**

Key Words: forkhead transcription factors ■ neointima ■ p27 ■ vascular smooth muscle cell

Despite the recent advances in strategies to prevent neointimal growth after angioplasty, it still remains the major limitation of percutaneous coronary interventions.[1] Although the pathogenic mechanism of restenosis is complex, the proliferation and migration of vascular smooth muscle cells (VSMCs) after balloon injury seems to be a major factor in this process.[2–4] Therefore, we along with many investigators have targeted VSMC proliferation and migration as a means to counter restenosis.[5,6]

FKHRL1 (forkhead transcription factor in rhabdomyosarcoma-like 1, FOXO3a) is a member of the forkhead transcription factor family, which is emerging as a key factor in pathways that regulate differentiation, metabolism, proliferation, and survival of cells.[7] FKHRL1 is negatively regulated by the PI3K/Akt pathway.[8–10] Phosphorylation of FKHRL1 by Akt leads to cytoplasmic retention and impairment of its nuclear transcriptional activity. Past studies have shown that the key actions of forkhead family transcription factors are cell-cycle arrest[11–14] and apoptosis.[9]

Taken together, FHKRL1 may affect the cell cycle and apoptosis of VSMCs, and may serve as a therapeutic target to inhibit neointimal growth after angioplasty. In the present study, we examined the effects of FKHRL1 on VSMC survival and proliferation in vitro. We also investigated whether FKHRL1 plays a role in neointima formation after balloon injury, and we tested whether the cytotoxic and cytostatic properties FKHRL1 can reduce neointimal hyperplasia after balloon injury in a rat carotid injury model.

Materials and Methods

Construction of Adenoviral Vectors Expressing Constitutively Active Triple Mutant

Detailed preparation procedures were previously described.[11] The hemagglutinin-tagged human FKHRL1 triple mutant (TM: T32A/

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S253A/S315A) and wild-type sequence cDNA were subcloned into a shuttle vector pAdTrack–cytomegalovirus, which contains green fluorescent protein (GFP) under the control of a separate cytomegalovirus promoter. The FKHRL1-AAA triple mutant (FKHRL1-TM) is not phosphorylatable because 3 phosphorylation sites, Thr32, Ser253, and Ser315, were replaced by alanine residues. For experiments with these reagents, control cultures were infected with an adenoviral vector expressing only the GFP transgene (Ad-GFP) prepared by the same system.

Cell Culture

Cells were incubated at 37°C in Dulbecco modified Eagle medium containing 10% fetal bovine serum with penicillin/streptomycin. Primary cultures of rat smooth muscle cells were prepared from thoracic aortas of adult male Sprague–Dawley rats according to Mader et al.[12] For all experiments, rat aortic SMCs from passages 6 to 7 were used. SMCs were grown to 70% to 80% confluence and then made quiescent by incubation with Dulbecco modified Eagle medium containing 0.1% fetal bovine serum for 48 hours

Cell Viability Assays

Cell viability was determined by WST-1 assay (Roche) as suggested by the manufacturer. Soon after, VSMCs were seeded in 96-well plates and infected with the indicated adenoviral vectors. At 24 and 48 hours after infection, 10 μL Cell Proliferation Reagent WST-1 was added and incubated for 4 hours at 37°C. Absorbency was measured using an enzyme-linked immunosorbent assay reader at 440 nm

FKHRL1 and Apoptosis

Subconfluent rat VSMCs were infected with adenoviruses (Adv-FKHRL1-TM and Adv-GFP, respectively) at 50 multiplicities of infection (moi) for 48 hours, harvested with trypsin, fixed in cold 90% ethanol for 20 minutes, and then resuspended in staining buffer consisting of 1 mg/mL RNaseA, 20 μg/mL propidium iodide, and 0.01% NP40. DNA content was analyzed by flow cytometry on FL-2 channel and gating was set to exclude debris and cellular aggregates. For each analysis, 10 000 events were counted.

FKHRL1 and Cell-Cycle Regulation

To test the effect of FKHRL1 on cell cycle, rat VSMCs were starved without serum and simultaneously infected with adenoviruses, Adv-FKHRL1-TM and Adv-GFP respectively, at 50 moi for 24 hours, and stimulated with 20% fetal bovine serum for 24 hours. After serum stimulation, cells were trypsinized, centrifuged at 1200 rpm for 5 minutes, washed with phosphate-buffered saline, and treated with RNase A (20 μg/mL). DNA was stained with propidium iodide (100 μg/mL) for 30 minutes at 4°C in the dark. DNA content of the cells was analyzed using a Becton Dickinson fluorescence-activated cell sorter Calibur flow cytometer (Becton Dickinson, San Jose, Calif). DNA histogram analysis was performed using ModFitLT software (Becton Dickinson). The percentage of each cell cycle phase was compared between 2 groups.

Immunoblot Assay

Cells were infected with adenoviral vector (Adv-FKHRL1-TM or Adv-GFP) at 50 moi for 20 hours. Cells were washed in phosphate-buffered saline and harvested by scraping in 50 mmol/L Tris-HCl (pH 7.2), 250 mmol/L NaCl, 1% NP40, 0.05% SDS, 2 mmol/L EDTA, 0.5% deoxycholic acid, 10 mmol/L glycerophosphate, and 1 mmol/L phenyl methyl sulfonyl fluoride. The primary antibodies used were anti-total FKHRL1 antibody (1:500 dilution; Upstate Biotechnology), anti-HA (hemagglutinin; 1:4000; Roche), and anti-p27 (1:1000; BD Transduction Laboratories).

Measurements of phospho-FKHRL1 and p27 in Carotid Arteries After Balloon Injury

A previously well-established rat carotid artery balloon injury model was used.[5] The vessel was harvested at several serial points after balloon injury (3 hours, 24 hours, 72 hours, and 2 weeks) to examine the endogenous change in FKHRL1 phosphorylation and p27 expression. Western blot analysis of vessel protein lysates using anti-phospho-FKHRL1 antibody (1:100; Upstate Biotechnologies) and anti-p27 antibody (1:100; Santa Cruz) were performed. The serial change in the expression of phopho-FKHRL1 and p27 were also confirmed by immunohistochemistry using phospho-FKHRL1 antibody (1:50; Upstate Biotechnologies) and anti-p27 antibody (1:50; Santa Cruz).

Adenoviral Vector-Mediated Gene Delivery After Rat Carotid Artery Balloon Denudation Injury

An adenoviral vector, expressing either the nonphosphorylatable, constitutively active of FKHRLl (FKHRL1-TM), or the control gene, GFP, were used for gene delivery. Forty-three adult male Sprague–Dawley rats weighing from 400 to 500 grams (Daehan Biolink Co) were anesthetized with a combination anesthetic (ketamine, 70 mg/kg; xylazine, 7 mg/kg intraperitoneal; Yuhan Corp, Bayer, Korea). The balloon injury was performed in a common carotid artery by 2-French catheter as previously described.[5] After balloon injury, viral infusion mixtures with 5×10^8 pfu of virus containing either constitutively active FKHRL1 gene (Adv-FKHRL1-TM) or control gene (Adv-GFP) diluted to a total volume of 100 μL were instilled into the arterial segment that was isolated by vascular clamps and incubated for 30 minutes.

Morphometric Analysis

The vessels of rats were harvested on days 3 and 14 after balloon injury as previously described.[5] The tissues were then embedded in paraffin, and sections were stained with hematoxylin and eosin or elastica van Gieson. The extent of neointimal formation was quantified by computed planimetry of histologically stained sections. The cross-sectional areas of the blood vessel layers including the lumen area, intimal area, and medial area were quantified at 3 different sections (proximal, middle, and distal, n=10) by using Image Pro Plus Analyzer Version 4.5 (Media Cybernetics). The intima-to-media ratios were calculated from the mean of these determinations. In addition, the number of nucleated cells in the media was counted at day 3 after balloon injury from 4 different high-power fields per sample at 3 levels (n=8).

Immunohistochemical Staining

To detect proliferating cells, immunohistochemical staining against proliferating cell nuclear antigen (PCNA) were performed on the virus-treated arteries.[5] Proliferation was assessed by quantifying the percentage of PCNA-positive cells against total nucleated cells in 4 different sectors per tissue section (n=8). Apoptotic cells were confirmed by treating specimens with 1:200 diluted cleaved caspase-3 antibody (Cell Signaling Technology) in a similar manner. The transduction efficiency was determined as the percentage of hemagglutinin (which was tagged to the adenoviral vector) stained versus total medial and neointimal cells per arterial section. The calculations were performed from 4 different high-power fields per sample, which were cut at 3 different levels of the carotid artery (n=8).

Double Immunohistochemical Staining

For double-staining we used an EnVision Doublestain kit (DakoCytomation) according to the manufacturer's instructions. Briefly, the first part of the 2-part procedure was performed using primary antibody (anti-α smooth muscle actin antibody, 1:100; DAKO), peroxidase-labeled polymer, and liquid DAB. After initial staining with liquid DAB, a double-staining block was added to the slides for 3 minutes. Then, the second part of the procedure was performed using primary antibody (anti-HA antibody, 1:250; Santa Cruz; or anti-caspase-3 antibody, 1:100; Cell Signaling Technology), alkaline phosphatase-labeled polymer, and fast red. After fast red staining, hematoxylin (DAKO) counterstaining was performed.

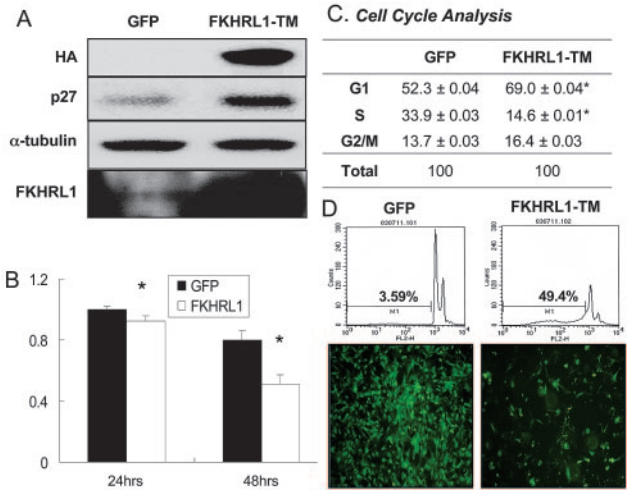

Figure 1. Effect of constitutively active FKHRL1-TM gene transfer on VSMCs in vitro. A, Immunoblot analysis of harvested cells 24 hours after gene delivery. B, WST-1 assays performed at 24 and 48 hours after gene transfer to cultured VSMCs in Dulbecco modified Eagle medium with fetal bovine serum 10% showing significant decrease in cell viability after overexpression of FKHRL1 (*$P<0.01$). C, Fluorescence-activated cell sorter analysis of the cell cycle showing a typical profile of G0/G1 cell-cycle arrest in the FKHRL1 group. D, Increased apoptosis after FKHRL1 overexpression as examined by fluorescence-activated cell sorter analysis and fluorescent microscopy. The construct of adeno-FKHRL1 also contains the GFP gene.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labeling Staining

Detection of apoptotic cells in vivo was also performed using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)[13] method with minor modifications. Apoptotic cells were quantified by counting the percentage of TUNEL-positive cells against total nucleated cells in 4 different sectors per tissue section (n=8).

Statistical Analysis

All data were presented as mean±SD. Comparisons between constitutively active FKHRL1 transfected and control group were performed using an unpaired 2-tailed *t* test, but the comparison of results from in vitro studies were performed using Mann–Whitney *U* test because of small numbers. Values of $P<0.05$ were considered significant. SPSS 11.0 software was used for all statistical calculations.

Results

FKHRL1 Overexpression Induces p27 Expression in Cultured VSMCs

Efficient transfection of constitutively active FKHRL1-TM gene was confirmed by detecting the expression of hemagglutinin, which was tagged onto the viral construct, as shown by Western blot analysis (Figure 1A; cells harvested 24 hours after gene delivery). The overexpression of FKHRL1-TM increased the expression of p27 compared with control. In addition, we confirmed the existence of endogenous FKHRL1 expression in rat VSMCs using anti-FKHRL1 Ab (Figure 1A).

FKHRL1 Overexpression Decreases VSMC Viability: Effect of Cell-Cycle Arrest and Apoptosis

To investigate the effect of FKHRL1 overexpression on VSMC viability, serum-stimulated (fetal bovine serum 10%) rat VSMC cultures were transfected with either Adv-GFP or Adv-FKHRL1-TM at 50 moi, which results in >95% transfection efficiency. Cell viability was measured 24 and 48 hours after gene transfer using WST-1 assay. Overexpression of FKHRL1 significantly diminished cell viability at both time points (Figure 1B; $P<0.01$, at both 24 and 48 hours).

Cell-cycle analysis using fluorescence-activated cell sorter (Figure 1C) showed that the percentage of cells in the G1 phase was significantly greater (52.3±0.04 versus 69.0±0.04%, n=4; $P=0.02$) and the percentage of cells in the S phase was significantly lower (33.9±0.03 versus 14.6±0.01%, n=4; $P=0.02$) in FKHRL1-TM–transfected VSMCs compared with GFP-transfected VSMCs. Thus, the G1/S ratio was significantly greater in the FKHRL1-TM group, consistent with cell-cycle arrest of the G0/G1 phase.

To assess the effect of FKHRL1 overexpression on VSMC apoptosis, fluorescence-activated cell sorter analysis of apoptotic bodies with hypodiploid DNA was performed. Adv-FKHRL1-TM gene transfer resulted in significantly increased subdiploid fraction (sub G1 peak) compared with GFP, confirming increased apoptosis. Under fluorescent microscopy, FKHRL1-TM gene transfer significantly increased floating dead cells with condensed and fragmented nuclei compared with GFP control at 48 hours (Figure 1D).

Forkhead Transcription Factor Is Phosphorylated Immediately After In Vivo Balloon Injury

From the in vitro data, we concluded that the overexpression of FKHRL1 leads to VSMC apoptosis and cell-cycle arrest. Because the proliferation of VSMCs after balloon injury is an important mechanism of neointima formation after angioplasty, we hypothesized that overexpression of FKHRL1 might reduce neointima formation. Before testing this hypothesis, we examined the endogenous expression of FKHRL1 and its downstream effecter molecule, p27, after balloon injury. Western blot of in vivo vessel samples at various time points showed that phosphorylation of FKHRL1 resulting in its inactivation occurs as soon as 3 hours after balloon injury, decreases at 24 hours, and then returns to baseline levels by day 3. The expression of p27, which we expected to be downregulated by phosphorylation of FKHRL1, showed a temporal delay in comparison to its upstream transcription factor FKHRL1. Compared with phospho-FKHRL1, which was markedly increased at 3 hours, the expression of p27 was similar to baseline up to 3 hours after injury, slowly declining to its lowest expression at day 3 (Figure 2A). The changes observed with Western blot samples were well-correlated with the immunohistochemistry results of phospho-FKHRL1 (Figure 2B) and p27 (Figure 2C).

Constitutively Active FKHRL1-TM Gene Transfer Increases p27 and Inhibits Neointima Formation After Balloon Injury

To examine the effect of FKHRL1 overexpression on neointimal formation after angioplasty, we delivered adenoviral vectors containing constitutively active FKHRL1-TM to balloon-injured rat carotid arteries. Successful gene transfer of FKHRL1 was confirmed in the vasculature by detecting the hemagglutinin tag using Western blot analysis. The gene transduction efficiency, which was measured as the percent-

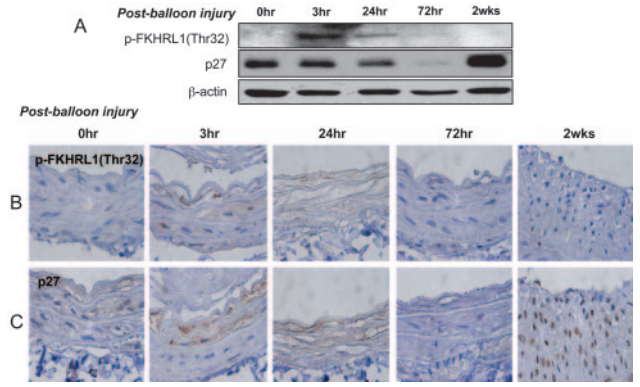

Figure 2. Endogenous serial expression of phospho-FKHRL1 and p27 after balloon injury. A, Western blot showing serial change in expression of phospho-FKHRL1 and p27 in vessel samples after balloon injury. The response of p27 expression, which is downstream of FKHRL1, shows a temporal delay compared with phospho-FKHRL1. B and C, Corresponding immunohistochemistry of phospho-FKHRL1 and p27. Note that staining is limited to the cytoplasm showing inhibited nuclear translocation of FKHRL1.

age of hemagglutinin (which was tagged to the adenoviral vector) stained versus total medial and neointimal cells per arterial section, was 38.6%±10.7% (n=8). In the control GFP group, the expression of p27 was low at day 3 and increased thereafter at day 14. However, the gene transfer of constitutively active FKHRL1 resulted in a significant increase in p27 expression compared with GFP at day 3. Although the expression of hemagglutinin was faint at week 2, the higher expression of p27 in the FKHRL1-TM group was sustained (Figure 3A). Immunoprecipitation Western blotting of total FKHRL1 showed that endogenous total FKHRL1 expression did not change after balloon injury and was only significantly increased after FKHRL1-TM gene transfer (Figure IA, available online at http://atvb.ahajournal-

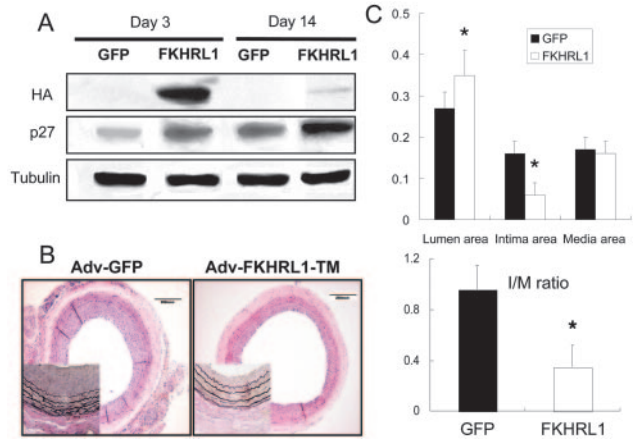

Figure 3. Effect of FKHRL1-TM gene transfer on neointima formation after balloon injury. A, Western blot confirming efficient delivery of the FKHRL1-TM transgene and increased expression of p27 at days 3 and 14. The expression of p27 is greatly increased by FKHRL-TM gene transfer at day 3 compared with the control group. Although p27 expression was increased in the GFP group at 2 weeks compared with day 3, the FKHRL1-TM group still maintained higher p27 expression at 2 weeks compared with the GFP control group. B, Hematoxylin and eosin and Elastica van Gieson (in set) staining of artery at 14 days after balloon injury. C, Morphometry at 14 days after gene delivery.

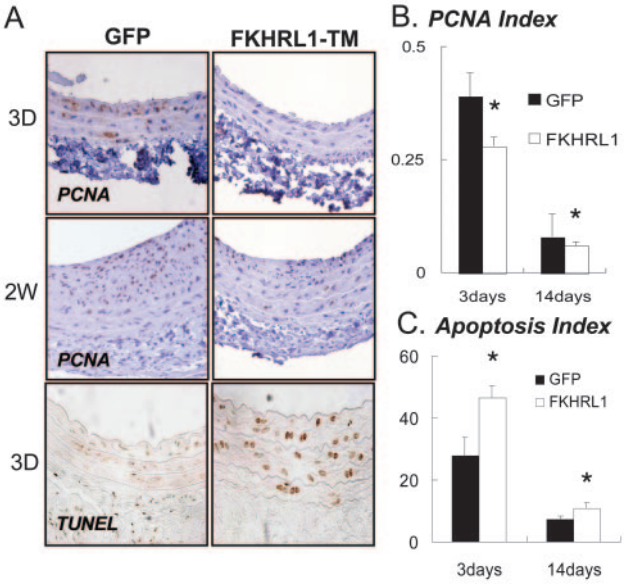

Figure 4. Effect of FKHRL1-TM gene transfer on VSMC proliferation and apoptosis after balloon injury. A, Immunohistochemical staining against PCNA at days 3 and 14 and TUNEL staining at day 3 showing markedly reduced PCNA-positive cells at both time points and increased apoptotic cells in the active FKHRL1-transfected group. B, Proliferative index. C, Apoptotic activity

s.org). Furthermore, the cells transduced with the adenoviral vectors were confirmed as medial smooth muscle cells by double immunohistochemical staining (Figure IB). Morphometric analysis 14 days after gene delivery revealed that the overexpression of the constitutively active FKHRL1 gene resulted in a significantly reduced neointimal area (0.16±0.03 versus 0.05±0.03 mm^2; $P<0.01$), whereas there was no significant difference in medial area (0.17±0.03 versus 0.16±0.03 mm^2; $P=0.34$) compared with the GFP-transfected group (Figure 3B and 3C). Although there were no significant differences in medial area, the cellularity of the media was significantly lower in the FKHRL1-TM–transfected group at day 3 (42.4±5.3 versus 28.9±6.1 cells/high-power field, for control versus FKHRL1-TM, n=8; $P<0.01$). This led to a significantly lower intima-to-media ratio (0.96±0.19 versus 0.35±0.17; $P<0.01$) and a greater lumen area (0.27±0.04 versus 0.35±0.06 mm^2; $P<0.01$) compared with the GFP-transfected group (Figure 3B and 3C).

Constitutively Active FKHRL1-TM Gene Transfer Inhibits Proliferation and Increases Apoptosis of VSMCs After Balloon Injury

Immunohistochemical staining for PCNA was performed to examine the effects of the constitutively active FKHRL1 gene transfer on VSMC proliferation during neointimal formation after balloon injury. PCNA-positive cells were markedly reduced in the FKHRL1-TM transfected group (Figure 4A). The proliferative index (calculated as the fraction of positive cells among total nucleated cells) was significantly lower in the FKHRL1-TM gene-transfected group at both 3 days (0.38±0.05 versus 0.28±0.05, n=8, $P=0.001$ for GFP versus FKHRL1-TM) and 2 weeks (0.08±0.19 versus 0.06±0.01, n=8, $P=0.02$ for GFP versus FKHRL1-TM) after gene delivery (Figure 4B).

To determine the effect of constitutively active FKHRL1 gene transfer on apoptosis after balloon injury in vivo, TUNEL staining (Figure 4A) and immunohistochemistry for active and cleaved caspase-3 (Figure IIA, available online at http://atvb.ahajournals.org) was performed at 3 days after balloon injury. The apoptotic activity of VSMCs was high in the GFP-transfected and FKHRL1-TM–transfected group, although apoptotic activity was higher in the FKHRL1-TM–transfected group (27.9±5.88 versus 46.5±4.05%, n=8, $P<0.001$ for GFP versus FKHRL1-TM). Even at 2 weeks after balloon injury, when the apoptotic activity declined considerably, the apoptotic activity remained significantly higher in the FKHRL1-TM–transfected group compared with the GFP-transfected group (7.3±1.11% versus 10.9±1.78%, n=8, $P<0.001$ for GFP versus FKHRL1-TM) (Figure 4C). Furthermore, double immunohistochemistry showed that the cells undergoing apoptosis and thus positive for caspase 3 are smooth muscle cells (Figure IIB).

Discussion

The process of neointima formation after balloon injury is complex, and elucidation of possible key molecules involved in the pathophysiology of neointimal hyperplasia may help in the efforts to reduce restenosis. In the present study, we report for the first time to our knowledge that FKHRL1 and p27 play an important role in the response to balloon denudation injury and neointima formation in rats. In vitro, the overexpression of FKHRL1 by constitutively active FKHRL1-TM gene transfer resulted in increased apoptosis, induction of p27, and G1/S phase cell-cycle arrest of cultured VSMCs. In vivo, carotid balloon injury resulted in phosphorylation of FKHRL1, which led to temporally delayed p27 downregulation and neointimal formation in rat carotid arteries. The importance of FKHRL1 and p27 were confirmed by constitutively active FKHRL1 gene transfer, which increased p27 expression and resulted in decreased neointimal hyperplasia after angioplasty.

FKHRL1 (forkhead transcription factor in rhabdomyosarcoma like 1, FOXO3a) is a member of the forkhead transcription factor family, which has been shown to play an important role in cell-cycle arrest and apoptosis of various cells. In various cancer cell lines,[14,15] endothelial cells,[16] and VSMCs,[17] forkhead transcription factors have been shown to induce G1 phase cell-cycle arrest. Recently, several studies demonstrated the regulation of p27 by forkhead transcription factors. Forkhead transcription factors were shown to mediate cell-cycle regulation by PKB through p27 but not p21 in cancer cell lines,[14,15] and the inhibition of FKHRL1 was reported to promote endothelial proliferation by downregulation of p27 in human endothelial cells.[16] In VSMCs, inactivation of forkhead transcription factors by mechanical strain resulted in transcriptional downregulation of p27.[17]

In addition, FHKRL1 has been shown to induce apoptosis in neuronal cell lines and fibroblasts by upregulation of Fas-ligand expression and activation of the death receptor pathway.[9] However, in hematopoietic cells, the "intrinsic" mitochondrial pathway, rather than the "extrinsic" Fas-mediated pathway, was shown to promote apoptotic cell death after activation of FKHRL1 signaling.[18] In VSMCs, we previously showed that FKHRL1 activates caspase 9, caspase 3, and c-Jun sequentially, and induces FasL to result in apoptosis under conditions of cellular stress.[19] In the present study, overexpression of FKHRL1 resulted in increased expression of p27 and decreased survival of cultured VSMCs, which was caused by increased apoptosis and cell-cycle arrest. Constitutively active FKHRL1 gene transfer resulted in a marked increase in apoptosis and a typical profile of G0/G1 phase cell-cycle arrest (increase in cells in G1 phase and decrease in cells in S phase).

The $p27^{kip1}$ is a cyclin-dependent protein kinase (CDK) inhibitor, which regulates the cell cycle via downregulation of CDK2 activity and repression of cyclin A transcription.[20] CDK/cyclin holoenzymes facilitate the progression of the cell cycle and is negatively regulated by the interaction with specific CDK inhibitory proteins that cause cell-cycle arrest when overexpressed in transformed and nontransformed cell lines.[21–23] Mice lacking the CDK inhibitor function of p27 display enhanced growth and multiple organ hyperplasia[24] and overexpression of p27 in rat carotid arteries attenuated neointimal hyperplasia.[25] In past studies, it has been shown that p27 expression is induced after balloon injury. In addition, the importance of p27 in regulating neointimal hyperplasia is well-documented by a previous study, which demonstrated that p27 gene transfer attenuates neointimal hyperplasia,[26] and by the success of rapamycin-coated stent in reducing restenosis after coronary angioplasty.[27]

The sequential change in expression levels of p27 after vessel injury seems to be somewhat different among different species. Reis et al reported that p27 began to increase 48 hours after balloon injury and persisted through the fourth week in mouse femoral artery injury model,[28] whereas in rat carotid arteries, p27 was already increased at 48 hours after balloon injury.[26] In porcine artery injury model, p27 began to decrease after balloon injury, stayed at the bottom level from day 4 to day 14, and then began to increase.[29] In the present study, we found that there is a temporal delay between the phosphorylation of FKHRL1 and the consequent downregulation in p27 expression. Compared with FKHRL1 phosphorylation, which is strongest at 3 hours after injury and declines to undetectable baseline levels by day 14, p27 expression only starts to decline slightly at 3 hours after balloon injury and reaches nadir at 72 hours, finally to be strongly re-expressed at day 14. The increase from baseline and decrease thereafter of phospho-FKHRL1 coincides with the downregulation from baseline and upregulation thereafter of p27 except with a temporal delay. This sequential change is concordant with previous cell kinetic studies which showed that VSMC proliferation and migration starts early after vessel injury, is greatest at ≈72 hours, and slowly declines to baseline levels by 2 weeks. Furthermore, we found that the gene transfer of constitutively active FKHRL1 results in upregulation of p27 and significant inhibition of neointimal proliferation after angioplasty, suggesting the importance of FKHRL1 phosphorylation and consequent downregulation of p27 in neointima formation. This is a novel finding that may have implications in future research. In the FKHRL1-TM gene transfer group, FKHRL1-TM was strongly expressed at day 3, whereas only faintly expressed at 2 weeks. Expression of p27, however, was moderate at day 3 and strong at 2 weeks, although its expression at each time point (3 days and 2 weeks) was significantly greater in the FKHRL1-TM group

than that of the control group. The disparity is probably because of the fact that after adenovirus-mediated gene transfer, the expression of the adenoviral transgene is highly expressed at 3 days and declines markedly to low levels by 2 to 3 weeks.[30,31] Considering also that a significant portion of the gene-transfected smooth muscle cells underwent apoptosis, the faint expression of FKHRL1-TM at 2 weeks is not surprising. The high expression of p27, however, was more because of the endogenous restoration of p27 expression in VSMCs, as we showed in Figure 2A. As seen in Figure 2A, the rebound expression of p27 at 2 weeks after injury is even stronger than at baseline levels.

In conclusion, balloon angioplasty leads to the phosphorylation of FKHRL1 and downregulation of p27 resulting in a proliferative phenotype in VSMCs. FKHRL1 overexpression increases p27 expression, leading to G1/S phase cell-cycle arrest and increased apoptosis of VSMCs, thereby inhibiting neointimal hyperplasia. This study reveals the importance of FKHRL1 in proliferation and viability of VSMCs and suggests that it may serve as a target for interventions to inhibit neointima formation after angioplasty.

Acknowledgments

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References

1. Topol EJ, Serruys PW. Frontiers in interventional cardiology. *Circulation*. 1998;98:1802–1820.
2. Ferns GA, Avades TY. The mechanisms of coronary restenosis: insights from experimental models. *Int J Exp Pathol*. 2000;81:63–88.
3. Schwartz RS, Murphy JG, Edwards WD, Camrud AR, Vliestra RE, Holmes DR. Restenosis after balloon angioplasty. A practical proliferative model in porcine coronary arteries. *Circulation*. 1990;82:2190–2200.
4. Clowes AW, Reidy MA, Clowes MM. Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. *Lab Invest*. 1983;49:327–333.
5. Park KW, Yang HM, Youn SW, Yang HJ, Chae IH, Oh BH, Lee MM, Park YB, Choi YS, Kim HS, Walsh K. Constitutively active glycogen synthase kinase-3{beta} gene transfer sustains apoptosis, inhibits proliferation of vascular smooth muscle cells, and reduces neointima formation after balloon injury in rats. *Arterioscler Thromb Vasc Biol*. 2003;23:1364–1369.
6. Yang HM, Kim HS, Park KW, You HJ, Jeon SI, Youn SW, Kim SH, Oh BH, Lee MM, Park YB, Walsh K. Celecoxib, a cyclooxygenase-2 inhibitor, reduces neointimal hyperplasia through inhibition of Akt signaling. *Circulation*. 2004;110:301–308.
7. Weigel D, Jurgens G, Kuttner F, Seifert E, Jackle H. The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the Drosophila embryo. *Cell*. 1989;57:645–658.
8. Paradis S, Ruvkun G. Caenorhabditis elegans Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor. *Genes Dev*. 1998;12:2488–2498.
9. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell*. 1999;96:857–868.
10. Shin I, Bakin AV, Rodeck U, Brunet A, Arteaga CL. Transforming growth factor beta enhances epithelial cell survival via Akt-dependent regulation of FKHRL1. *Mol Biol Cell*. 2001;12:3328–3339.
11. Skurk C, Maatz H, Kim HS, Yang J, Abid MR, Aird WC, Walsh K. The Akt-regulated forkhead transcription factor FOXO3a controls endothelial cell viability through modulation of the caspase-8 inhibitor FLIP. *J Biol Chem*. 2004;279:1513–1525.
12. Mader SL. Influence of animal age on the beta-adrenergic system in cultured rat aortic and mesenteric artery smooth muscle cells. *J Gerontol*. 1992;47:B32–B36.
13. Ansari B, Coates PJ, Greenstein BD, Hall PA. In situ end-labelling detects DNA strand breaks in apoptosis and other physiological and pathological states. *J Pathol*. 1993;170:1–8.
14. Medema RH, Kops GJ, Bos JL, Burgering BM. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature*. 2000;404:782–787.
15. Nakamura N, Ramaswamy S, Vazquez F, Signoretti S, Loda M, Sellers WR. Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN. *Mol Cell Biol*. 2000;20:8969–8982.
16. Potente M, Fisslthaler B, Busse R, Fleming I. 11,12-Epoxyeicosatrienoic acid-induced inhibition of FOXO factors promotes endothelial proliferation by down-regulating p27Kip1. *J Biol Chem*. 2003;278:29619–29625.
17. Sedding DG, Seay U, Fink L, Heil M, Kummer W, Tillmanns H, Braun-Dullaeus RC. Mechanosensitive p27Kip1 regulation and cell cycle entry in vascular smooth muscle cells. *Circulation*. 2003;108:616–622.
18. Dijkers PF, Birkenkamp KU, Lam EW, Thomas NS, Lammers JW, Koenderman L, Coffer PJ. FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity. *J Cell Biol*. 2002;156:531–542.
19. Suhara T, Kim HS, Kirshenbaum LA, Walsh K. Suppression of Akt signaling induces Fas ligand expression: involvement of caspase and Jun kinase activation in Akt-mediated Fas ligand regulation. *Mol Cell Biol*. 2002;22:680–691.
20. Schulze A, Zerfass K, Spitkovsky D, Middendorp S, Berges J, Helin K, Jansen-Durr P, Henglein B. Cell cycle regulation of the cyclin A gene promoter is mediated by a variant E2F site. *Proc Natl Acad Sci U S A*. 1995;92:11264–11268.
21. Morgan DO. Principles of CDK regulation. *Nature*. 1995;374:131–134.
22. Peter M, Herskowitz I. Joining the complex: cyclin-dependent kinase inhibitory proteins and the cell cycle. *Cell*. 1994;79:181–184.
23. Grana X, Reddy EP. Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). *Oncogene*. 1995;11:211–219.
24. Kiyokawa H, Kineman RD, Manova-Todorova KO, Soares VC, Hoffman ES, Ono M, Khanam D, Hayday AC, Frohman LA, Koff A. Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). *Cell*. 1996;85:721–732.
25. Chen D, Krasinski K, Sylvester A, Chen J, Nisen PD, Andres V. Down-regulation of cyclin-dependent kinase 2 activity and cyclin A promoter activity in vascular smooth muscle cells by p27(KIP1), an inhibitor of neointima formation in the rat carotid artery. *J Clin Invest*. 1997;99:2334–2341.
26. Chen D, Krasinski K, Sylvester A, Chen J, Nisen PD, Andres V. Down-regulation of cyclin-dependent kinase 2 activity and cyclin A promoter activity in vascular smooth muscle cells by p27(KIP1), an inhibitor of neointima formation in the rat carotid artery. *J Clin Invest*. 1997;99:2334–2341.
27. Gallo R, Padurean A, Jayaraman T, Marx S, Roque M, Adelman S, Chesebro J, Fallon J, Fuster V, Marks A, Badimon JJ. Inhibition of intimal thickening after balloon angioplasty in porcine coronary arteries by targeting regulators of the cell cycle. *Circulation*. 1999;99:2164–2170.
28. Reis ED, Roque M, Cordon-Cardo C, Drobnjak M, Fuster V, Badimon JJ. Apoptosis, proliferation, and p27 expression during vessel wall healing: time course study in a mouse model of transluminal femoral artery injury. *J Vasc Surg*. 2000;32:1022–1029.
29. Tanner FC, Yang ZY, Duckers E, Gordon D, Nabel GJ, Nabel EG. Expression of cyclin-dependent kinase inhibitors in vascular disease. *Circ Res*. 1998;82:396–403.
30. Schulick AH, Dong G, Newman KD, Virmani R, Dichek DA. Endothelium-specific in vivo gene transfer. *Circ Res*. 1995;77:475–485.
31. Vassalli G, Agah R, Qiao R, Aguilar C, Dichek DA. A mouse model of arterial gene transfer: antigen-specific immunity is a minor determinant of the early loss of adenovirus-mediated transgene expression. *Circ Res*. 1999;86:e25–e32.