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Forkhead Transcription Factor FOXO3a Is a Negative Regulator of Angiogenic Immediate Early Gene CYR61, Leading to Inhibition of Vascular Smooth Muscle Cell Proliferation and Neointimal Hyperplasia

Hae-Young Lee,* Jae-Woong Chung,* Seock-Won Youn, Ju-Young Kim, Kyung-Woo Park, Bon-Kwon Koo, Byung-Hee Oh, Young-Bae Park, Brahim Chaqour, Kenneth Walsh, Hyo-Soo Kim

Abstract—Cysteine-rich angiogenic protein 61 (CYR61, CCN1) is an immediate early gene expressed in vascular smooth muscle cells (VSMCs) on growth factor stimulation, and its expression has been suggested to be associated with postangioplasty restenosis. The forkhead transcription factors are reported to play various roles in cellular proliferation, apoptosis, and even adaptation to cellular stress. We hypothesized that the forkhead transcription factor FOXO3a may regulate CYR61 expression in VSMCs and investigated the CYR61-modulating effect of FOXO3a in the process of vascular response to vasoactive signals and vascular injury. To evaluate the effect of FOXO3a on CYR61 expression, rat VSMCs were infected with adenoviral vectors expressing constitutively active FOXO3a (Ad-TM-FOXO3a). Constitutively active FOXO3a gene transduction suppressed CYR61 expression. Luciferase assay with the deletion constructs of the forkhead factor binding motif in CYR61 promoter region, which resulted in a significant decrease in luciferase expression compared with the intact construct, and chromatin immunoprecipitation analysis confirmed transcriptional regulation of CYR61 by FOXO3a. Serum and angiotensin II rapidly induced CYR61 expression, which was significantly reduced by Ad-TM-FOXO3a. Reduction of VSMC proliferation and migration associated with FOXO3a activation was significantly reversed by cotransfection of adenoviral vector expressing CYR61, whereas apoptosis induction by FOXO3a was not influenced. In a rat balloon carotid arterial injury model, CYR61 was rapidly induced in VSMCs in the early stage of injury and remained elevated until 14 days, which was suppressed by Ad-TM-FOXO3a transfection. After 14 days, there was a significant reduction in neointima by FOXO3a transduction compared with the control group (0.06 ± 0.02 versus 0.20 ± 0.07 mm², $P < 0.01$). Such reduction of neointimal hyperplasia by Ad-TM-FOXO3a was reversed by CYR61 replenishment. These data suggest that FOXO3a is a negative transcription factor of CYR61 and that suppression of CYR61 is among several mechanisms by which FOXO3a inhibits VSMC proliferation and neointimal hyperplasia. (*Circ Res.* 2007;100:372-380.)

Key Words: FOXO3a ■ CYR61 ■ Smooth muscle ■ Angiotensin II ■ Neointimal hyperplasia

Vascular smooth muscle cell (VSMC) proliferation and migration are characteristic features of various vascular pathologies, such as atherosclerosis and neointimal hyperplasia after vascular intervention.¹ Vasoactive growth factors including angiotensin II and mechanical stress are known to be involved in these processes.² Despite the recent advances in strategies to control restenosis, the regulatory mechanism has not been fully defined yet. Therefore, elucidation of possible key molecules involved in the pathophysiology of VSMC proliferation and migration following various stimuli may help controlling many vasculopathic conditions.

Cysteine-rich angiogenic protein 61 (CYR61, CCN1) is a cysteine-rich heparin binding protein that belongs to the novel CCN gene family.³ CYR61 was originally cloned as an immediate early gene expressed in fibroblasts after growth factor stimulation,⁴ and its expression has been reported to be associated with vascular restenosis, angiogenesis, and tumor growth.^{5,6} CYR61 was also reported to be expressed in VSMCs of atheromatous plaques, where its expression was regulated by angiotensin II.^{7,8}

During investigation of CYR61, we found that CYR61 contains the forkhead factor binding motif AAAGCAAACA⁹

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in its promoter region. This was interesting, because forkhead transcription factors are emerging as key players, under regulation of Akt, in roles ranging from cellular proliferation, migration, apoptosis, and even adaptation to cellular stress.¹⁰ Moreover, previous studies by our group¹¹ and others¹² have suggested a potential role of FOXO3a, the main forkhead transcription factor expressed in VSMCs, in preventing neo-intimal hyperplasia by cell cycle arrest and apoptosis induction.

The presence of the forkhead factor binding sequences in its promoter region, and its observed function in VSMCs, allowed the hypothesis that CYR61 might be regulated by FOXO3a. In this study, we tested this hypothesis and investigated the modulating effect of CYR61 by FOXO3a in the process of vascular response to vasoactive signals such as angiotensin II and vascular injury.

Materials and Methods

VSMC Culture, Gene Transfer Using Adenoviral Vectors, and Pharmacological Stimulation

Primary cultures of VSMCs were prepared from thoracic aortas of adult male Sprague–Dawley rats as previously described.¹³ Six- to 7-passage VSMCs were incubated at 37°C in DMEM containing 10% FBS (Cambrex) with 1% antibiotic/antimycotic solution (GIBCO BRL).

To evaluate the role of CYR61, adenovirus vector expressing CYR61 was used.¹⁴ In terms of FOXO3a, an adenoviral vector expressing constitutively active FOXO3a (Ad-TM-FOXO3a) was used.¹⁵ Briefly, Ad-TM-FOXO3a was constructed by replacing 3 phosphorylation sites, Thr32, Ser253, and Ser315, with alanine residues, a process that prevented phosphorylation by Akt. For blocking experiments, an adenoviral vector expressing dominant-negative form (Ad-DN-FOXO3a), the transactivation domain of which was deleted from the C terminus, or an adenoviral vector containing antisense FOXO3a cDNA was used. As a control, an adenoviral vector expressing green fluorescent protein (Ad-GFP) was used.

For gene transduction, VSMCs were infected with indicated adenoviral vectors at 50 multiplicities of infection (mois) for 24 hours and reached a concentration with >90% transfection efficiency, as shown in our previous report.¹¹ For pharmacological stimulation, angiotensin II (Sigma, A9525) was used with a concentration of 10⁻⁷ mol/L.

RT-PCR and Immunoblot Analysis

Changes in RNA expression of CYR61 were determined by RT-PCR as previously described.¹⁶ Primers used were as follows: forward primer: 5'-TCT CGT TGC TGC TCA TGA AAT T-3'; reverse primer: 5'-TAG AGT GGG TAC ATC AAA GCT TCA G-3'. Total RNA was isolated by the TRIzol method (Invitrogen) and reverse transcribed using the reverse transcription system (Promega), and cDNA was amplified with CYR61 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Immunoblot analysis was performed by modification of the procedures described previously.¹⁷ The primary antibodies used were as follows: anti-FOXO3a (rabbit polyclonal IgG, Upstate Biotechnology, 1:1000), anti-hemagglutinin (HA) (Roche Applied Science, 1:500), and anti-CYR61 (Santa Cruz Biotechnology, 1:500). As secondary antibody, anti-mouse IgG horseradish peroxidase (Promega) or anti-rabbit IgG HRP (Promega) was used at a dilution of 1:2000. Immunoblots were quantified using the chemiluminescent detection reagents ECL (Amersham).

Luciferase Promoter Activity Assay

Because the putative forkhead binding motif AAAGCAAACA⁹ exists from -760 to -751 in the promoter sequences of CYR61,¹⁸

the promoter region from -1093 to -209 upstream of translation start site (ATG) was isolated from human genomic DNA and subcloned into pGL3 basic luciferase expression vector (LUC) (Promega) at the *KpnI/XhoI* site to create a pGL3-CYR61-LUC (forward: 5'-cgg gta cc-TAA AGT GGG AAC CTC CA-3'; reverse: 5'-ccg ctc gag-TCT CGC TCG CGG TCT GCC-3'). And a deletion mutant was constructed as pGL3-ΔCYR61-LUC plasmid vector where the forkhead binding motif was deleted (from -565 to -209 upstream of ATG; forward: 5'-cgg gta cc-CTT CCC AAC ATA AGT CG-3'; reverse: 5'-ccg ctc gag-TCT CGC TCG CGG TCT GCC-3') (Figure 1D). A control plasmid expressing β-galactosidase was also transfected to serve as a reference and to control for the transfection efficiency in each experiment. After adenoviral infection, cells were incubated with 200 μL/well of reporter lysis buffer, and lysates were mixed with the luciferase substrate reagent. The absorbency was determined by a fluorometer (Lumi-Scint; Bioscan Inc), corrected for β-galactosidase activity determined by a standard assay (Promega) and reported as the relative activity compared with the control cells.

Chromatin Immunoprecipitation Assay

A chromatin immunoprecipitation assay was performed with a commercially available kit (Upstate Biotechnology) following the instructions of the manufacturer. Antibodies for HA and FOXO3a were used for immunoprecipitation of the DNA fragments. The DNA fragments were analyzed by PCR with primers specific for the CYR61 promoter (forward: 5'-CAG ATA ACT TGC CTC TCA CC-3'; reverse: 5'-TAC GAC TTA TGT TGG GAA GG-3').

Cell Viability Assay

Cell viability was measured using the WST-1 assay (Roche Molecular Biochemicals) according to the instructions of the manufacturer. VSMCs were seeded in 96-well plates at 2×10⁴ cells per well in DMEM (100 μL). After serum starvation for 24 hours, VSMCs were transfected with indicated adenoviral vectors (50 mois) for 24 hours. At the end of the incubation period, 10 μL of WST-1 was added and incubated for 4 hours at 37°C. Absorbency was measured using an ELISA reader at 440 nm.

Cell Proliferation and Apoptosis Assay

Cell proliferation and apoptosis were quantified by fluorescence-activated cell sorting. After infection with adenoviral vectors at 50 mois for 24 hours, cells were harvested, fixed, and analyzed by flow cytometry (Becton-Dickinson). For proliferation evaluation, histograms of DNA contents were analyzed using Modfit LT software (Verity Software) to characterize population fractions in each phase of the cell cycle. Apoptosis was determined by DNA fragmentation assessed by measuring the hypodiploid DNA contents.

Cell Migration Assay

To investigate the role of CYR61 on VSMC migration, we evaluated the effect of the supernatant, which was harvested from the culture dish of VSMCs transduced with indicated adenoviral vectors, on VSMC migration using the scratch-wound migration assay as described previously.¹⁹ In brief, VSMCs for the scratch assay were cultured to confluence on glass coverslips in DMEM with 10% FBS. Another set of VSMCs for supernatant harvest were cultured in 6-well plates. For supernatant collection, VSMCs in the 6-well plates were transfected with indicated adenoviral vectors (50 mois) for 24 hours, then 1 mL of the supernatant from each culture plate was collected. For the scratch assay, after scratching with a cell scraper to make the wound area, the collected supernatant was applied to VSMCs on the coverslips and incubated for 96 hours. The migration distance was quantified using Image-Pro 4.5 (Particle Engineering Research Center, University of Florida, Gainesville).

Rat Carotid Artery Balloon Denudation Injury

Fifteen adult male Sprague–Dawley rats weighing from 400 to 500 g (Daehan Biolink Co) were anesthetized with a combination anesthetic (ketamine, 70 mg/kg; xylazine, 7 mg/kg IP; Daehan Biolink

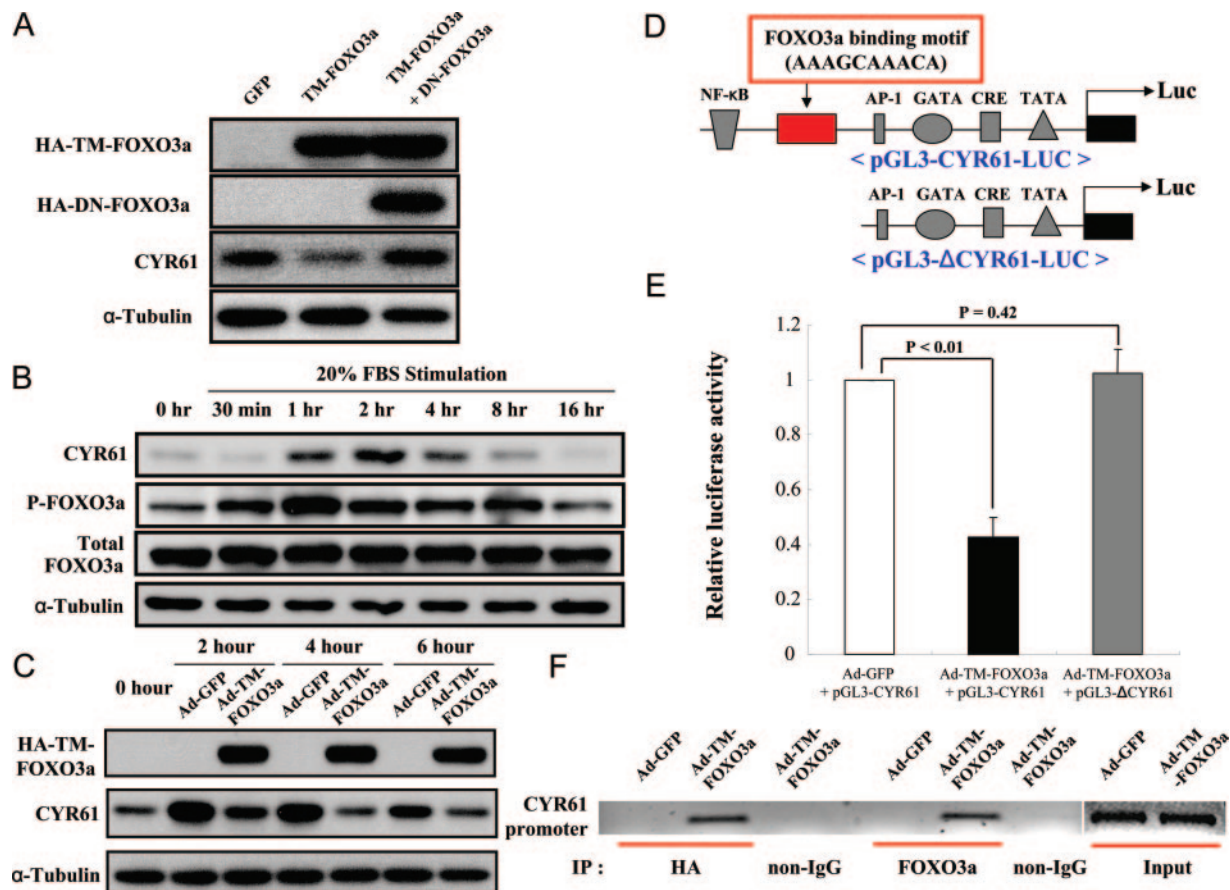


Figure 1. FOXO3a suppresses CYR61 expression in cultured rat VSMCs. **A**, Immunoblot analysis of a rat VSMC 24 hours after gene delivery. FOXO3a activation suppressed CYR61 expression, which was reversed by Ad-DN-FOXO3a cotransfection. **B**, Serial immunoblot analysis of CYR61 and phosphorylated (thus inactivated) native FOXO3a following serum (20% FBS) stimulation. **C**, Serial immunoblot analysis of CYR61 protein expression after adenoviral gene transfection. **D**, Sequence alignment of FOXO3a binding motif in CYR61 promoter region. Two types of plasmid vectors containing luciferase reporter gene were constructed as follows; pGL3-CYR61-LUC, plasmid with full sequence of CYR61 promoter; pGL3- Δ CYR61-LUC, plasmid with FOXO3a binding motif deleted. **E**, Relative luciferase activity compared with the control. Luciferase activity was normalized by β -galactosidase activity, which was cotransfected to normalize the plasmid transfection efficiency. The data represent means \pm SE (N=9). **F**, Chromatin immunoprecipitation assay of CYR61.

Co, Seoul, Korea). Balloon injury was performed in the common carotid artery by a 2-French catheter as previously described.¹¹ After balloon injury, viral infusion mixtures with 5×10^8 plaque-forming units of indicated adenoviral vectors diluted to a total volume of 50 μ L were instilled into the arterial segment that was isolated by vascular clamps and incubated for 30 minutes.

Morphometric Analysis

The vessels were harvested on days 3 and 14 after balloon injury, as previously described.²⁰ The extent of neointima formation was quantified by computed planimetry of 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=12) by using Image-Pro Plus Analyzer version 4.5 (MediaCybernetics). The intima-to-media ratios were calculated from the mean of these determinations.

Immunohistochemical Staining

Immunohistochemistry was performed as previously described.²⁰ To detect proliferating cells, immunohistochemical staining against proliferating cell nuclear antigen (PCNA) were performed.²¹ Proliferation was assessed by quantifying the percentage of PCNA-positive cells against total nucleated cells in 4 different sectors per tissue section (n=6).

Detection of apoptotic cells was also performed using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method, with minor modifications.²² Apoptosis was quantified by counting the percentage of TUNEL-positive cells against total nucleated cells in 4 different sectors per tissue section (n=6).

Statistics

All data are presented as mean \pm SD. Comparisons between groups were performed using the Mann-Whitney *U* test because of small numbers. A probability value of less than 0.05 was considered statistically significant.

Results

FOXO3a Suppresses CYR61 Expression in Cultured Rat VSMCs

Efficient transduction of constitutively active FOXO3a gene was confirmed by detecting HA, which was tagged onto the viral construct. The activation of FOXO3a suppressed the expression of CYR61, which was almost completely reversed by Ad-DN-FOXO3a (Figure 1A).

Next, we evaluated serial expression of CYR61 and phosphorylated (thus inactivated) native FOXO3a following se-

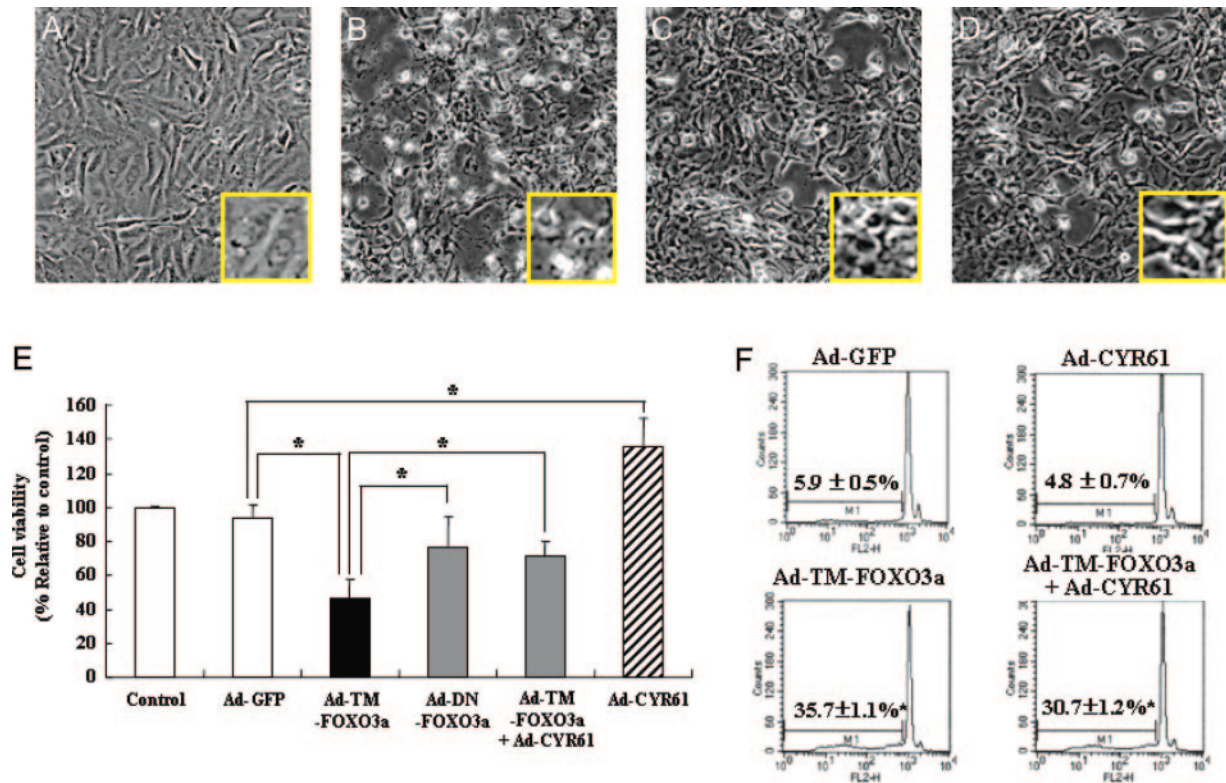


Figure 2. FOXO3a decreased VSMC viability: effect of CYR61 suppression on cell viability. A through D, Microscopic findings of VSMCs at 24 hours after transfection with 50 mois of indicated adenoviral vectors. The inset shows a magnified nucleus. A, VSMCs transfected with Ad-GFP; B, with Ad-TM-FOXO3a; C, cotransfected with Ad-TM-FOXO3a and Ad-DN-FOXO3a; D, cotransfected with Ad-TM-FOXO3a and Ad-CYR61. Magnification $\times 200$. E, WST-1 assay evaluating VSMC viability at 24 hours after adenoviral transfection. Bar graph illustrates cell viability of each group compared with the control (null treatment). F, Apoptotic fluorescence-activated cell sorting analysis measuring the hypodiploid DNA contents, showing significantly increased apoptotic fraction in the TM-FOXO3a-transfected group. Results are expressed as the mean \pm SEM. * $P < 0.05$ compared with the control ($n = 6$).

rum (FBS 20%) stimulation. Phosphorylation of FOXO3a was found to increase from 30 minutes and to peak at 1 hour after serum stimulation without change in total amount of FOXO3a. And CYR61 expression was found to increase from one hour and to reach its peak at two hours, the expression pattern of which was temporally well correlated with the change in FOXO3a phosphorylation (Figure 1B).

Then, we evaluated the modulation of CYR61 expression by FOXO3a. With serum stimulation, expression of CYR61 was swiftly increased, which was significantly reduced in TM-FOXO3a-transduced cells compared with the control (RT-PCR in Figure 1A in the online data supplement, available at <http://circres.ahajournals.org>; immunoblot in Figure 1C).

Finally, to confirm negative regulation of CYR61 by FOXO3a, we performed a promoter assay using a construct (pGL3-CYR61-LUC) in which the luciferase reporter gene is driven by the CYR61 promoter¹⁸ and a construct (pGL3- Δ CYR61-LUC) in which the 5'-forkhead binding motif was deleted (Figure 1D). VSMCs transfected with each CYR61 promoter construct were subsequently infected with Ad-TM-FOXO3a and assayed for luciferase activity. Ad-TM-FOXO3a transfection significantly suppressed the serum-induced luciferase activity driven by the CYR61 promoter in the pGL3-CYR61-LUC construct (luciferase activity, 0.43 ± 0.07 in Ad-TM-FOXO3a-transfected VSMCs, versus

1 ± 0 in Ad-GFP-transfected group, $n = 9$, $P < 0.01$). Such inhibitory effect of FOXO3a was not observed when forkhead binding motif was deleted out of the promoter region of CYR61 gene as in the pGL3- Δ CYR61-LUC construct (1.02 ± 0.09 , $P = 0.42$) (Figure 1E). The chromatin immunoprecipitation analysis also confirmed the transcriptional regulation of CYR61 by FOXO3a (Figure 1F).

FOXO3a Decreased VSMC Viability: Effect of CYR61 Suppression on Cell Cycle Arrest

Because we previously reported FOXO3a reduced VSMC viability through apoptosis induction and cell cycle arrest,¹¹ we investigated which aspect of CYR61 suppression contributed to the effect of FOXO3a activation. VSMCs were either transfected with Ad-TM-FOXO3a alone or cotransfected with Ad-CYR61 and cultured for 24 hours. We found that VSMCs transfected with Ad-TM-FOXO3a showed significant detachment from the culture plate and nuclear condensations consistent with apoptosis (Figure 2B), which were remarkably reduced by cotransfection with Ad-CYR61 (Figure 2D). Quantification of cell viability also showed that FOXO3a activation significantly reduced VSMC viability (cell viability relative to control (%), 46.4 ± 11.1 , versus 100 ± 12.2 , $n = 3$, $P < 0.05$), which was also significantly reversed by cotransfection with Ad-CYR61 ($71.2 \pm 8.2\%$, $n = 3$, $P < 0.05$ compared with Ad-TM-FOXO3a group, Figure

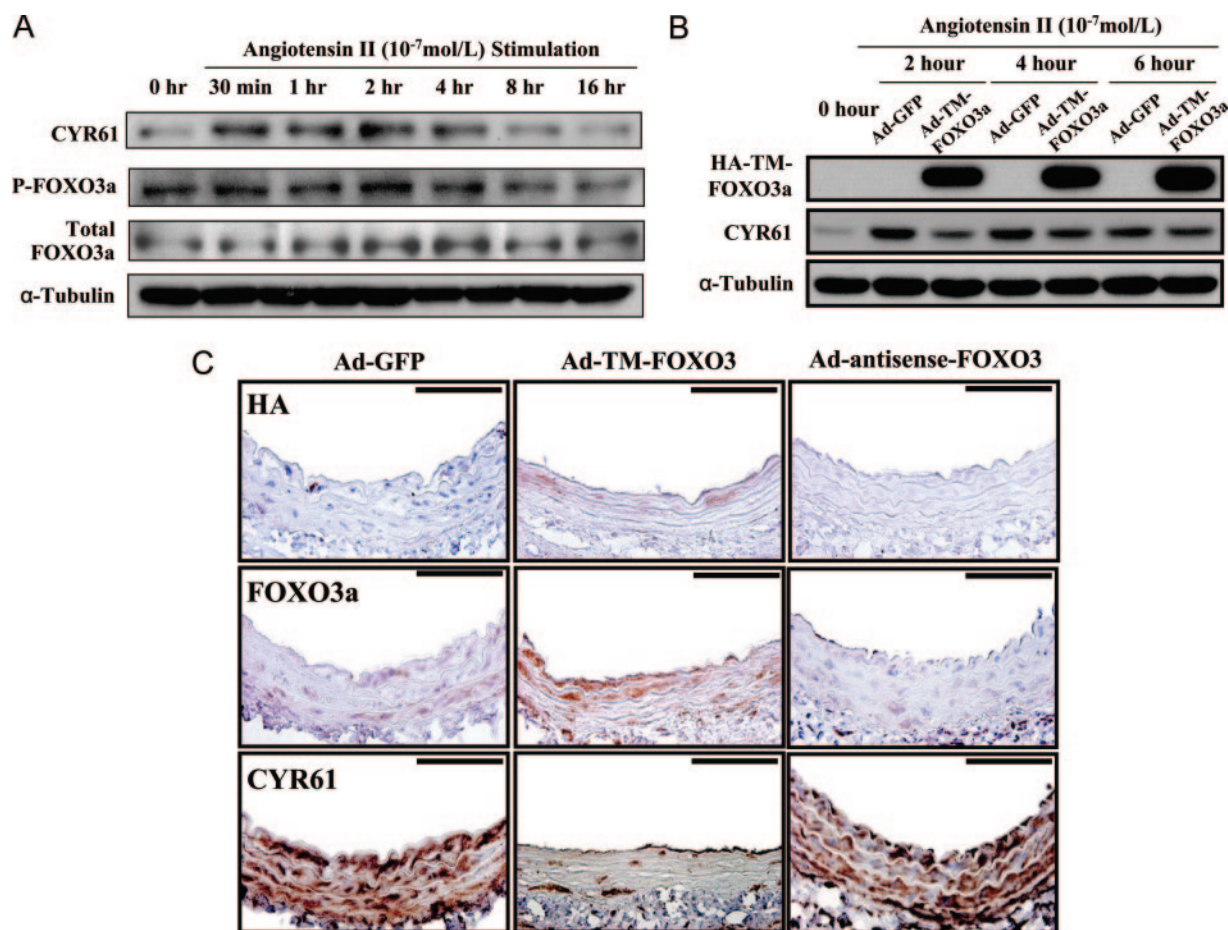


Figure 3. FOXO3a blocked angiotensin II-induced CYR61 expression. A, Serial immunoblot analysis of CYR61, phosphorylated (thus inactivated) native FOXO3a and total native FOXO3a after angiotensin II (10^{-7} mol/L) stimulation. B, Serial immunoblot analysis of CYR61 protein at 2, 4, and 6 hours after angiotensin II stimulation. C, Corresponding immunohistochemistry of FOXO3a and CYR61 2 hours after angiotensin II stimulation in rat aorta that were pretransfected with Ad-GFP (left), Ad-TM-FOXO3a (middle), and Ad-antisense-FOXO3a (right), respectively. Effective transfection of Ad-TM-FOXO3a and Ad-GFP to rat aorta was confirmed by staining for HA, whereas Ad-antisense-FOXO3a does not contain sequences for HA.

2E). Interestingly, we found Ad-CYR61 transfection alone increased viable cell count even compared with the control ($135.4 \pm 16.6\%$, $n=3$, $P<0.05$).

Next, we evaluated the role of CYR61 in apoptosis by apoptotic fluorescence-activated cell sorting analysis. FOXO3a activation significantly increased apoptotic fraction ($35.7 \pm 1.1\%$ versus $5.9 \pm 0.5\%$ in control, Figure 2F). However, Ad-CYR61 transfection did not show any significant effect on apoptosis either alone or when cotransfected with Ad-TM-FOXO3a.

Then, we evaluated the contributing role of CYR61 in cell proliferation. CYR61 transduction significantly propagated the cell cycle and increased the percentage of cells in the S phase either alone (40.0 ± 2.1 for Ad-CYR61 versus 24.0 ± 0.9 for the control, Ad-GFP, $P<0.05$) or when cotransfected with Ad-TM-FOXO3a ($33.3 \pm 2.0\%$ versus $25.2 \pm 1.2\%$ for Ad-TM-FOXO3a, $P<0.05$) (supplemental Table I).

Taken together, these data suggest that CYR61 suppression by FOXO3a activation contributed to the cell cycle arrest, although it did not contribute to apoptosis induction.

FOXO3a Blocks Angiotensin II-Induced CYR61 Expression

Angiotensin II is known to induce CYR61,⁷ and we investigated whether FOXO3a suppressed CYR61 expression even in a stimulated condition. On angiotensin II stimulation, CYR61 was rapidly induced from 30 minutes and peaked at 2 hours without change in the amount of either total or phosphorylated FOXO3a, indicating that CYR61 induction by angiotensin II is FOXO3a independent (Figure 3A). However, CYR61 induction by angiotensin II was significantly attenuated by TM-FOXO3a transduction in mRNA levels (supplemental Figure IB), as well as in protein levels (Figure 3B), suggesting that CYR61 expression was regulated by at least 2 different signal pathways, angiotensin II and FOXO3a.

Next, to confirm the regulation of CYR61 by FOXO3a, we made in vivo experiment by delivering adenoviral vectors containing TM-FOXO3a, antisense FOXO3a, or the control GFP to rat aortic rings. The gene transduction efficiency, which was measured as the percentage of cells positive for HA (which was tagged to the adenoviral vector), was

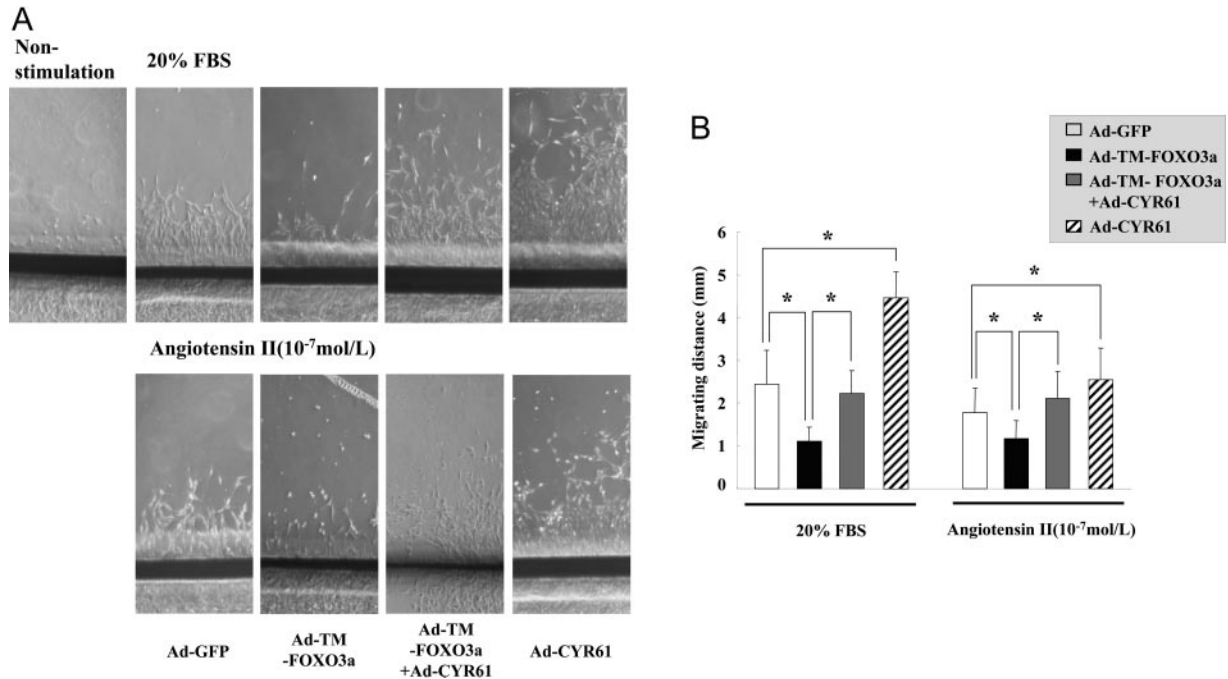


Figure 4. FOXO3a decreased VSMC migration: effect of CYR61 suppression. A, Scratch-wound migration assay. Microscopic findings of VSMCs at 96 hours after applied supernatant collected from culture dish of indicated adenoviral vector–transfected VSMCs in the presence either of 20% FBS (top panels) or of angiotensin II (10^{-7} mol/L). Magnification, $\times 100$. B, Bar graph illustrates migrating distance. $*P < 0.05$.

$41.4 \pm 4.3\%$ ($n=5$, Figure 3C, top). We incubated the aortic rings for 2 hours in DMEM with angiotensin II (10^{-7} mol/L) ex vivo. In the control vessel, the endogenous expression levels of FOXO3a were low and angiotensin II significantly induced CYR61. In Ad-TM-FOXO3a transfected blood vessels, however, large amounts of FOXO3a were expressed and CYR61 expression was significantly suppressed, even after angiotensin II stimulation. Finally, in Ad-antisense-FOXO3a–transfected blood vessels, the endogenous FOXO3a decreased and CYR61 expression increased more than the control vessel (Figure 3C).

FOXO3a Decreased VSMC Migration: Effect of CYR61 Suppression

Increased VSMC chemotaxis is another component causing neointimal hyperplasia.²³ We evaluated the role of FOXO3a and CYR61 in VSMC migration by the scratch-wound assay. To evaluate the effect of secreted CYR61, we used supernatant harvested from the culture dish of VSMCs transduced with indicated adenoviral vectors. Supernatant from Ad-TM-FOXO3a transfected VSMCs significantly decreased VSMC migration compared with the control, which was nearly completely reversed by cotransfection with Ad-CYR61 (Figure 4A and 4B). We also observed that supernatant from Ad-CYR61–transfected VSMCs significantly enhanced VSMC migration, even compared with the control, suggesting that CYR61 suppression contributed to decreased VSMC migration by FOXO3a activation.

FOXO3a Blocks CYR61 Expression After Balloon Injury in Rat Carotid Artery

We investigated the role of FOXO3a activation in CYR61 expression after balloon injury. We delivered the control

vectors, Ad-GFP, or Ad-TM-FOXO3a to rat carotid arteries immediately after balloon injury and evaluated CYR61 expression (Figure 5A). From day 3, CYR61 began to accumulate in media, and at day 14, CYR61 markedly accumulated, thus being detectable both in the media and in the proliferating neointima of the control vessels. Conversely, in TM-FOXO3a–transduced blood vessels, CYR61 expression was remarkably suppressed, even after balloon-injury, accompanied by remarkably less neointimal growth (Figure 5A). Then, we obtained total cell lysates from rat carotid artery and quantified CYR61 expression by tissue immunoblot, of which patterns were well correlated with those observed in immunohistochemistry (Figure 5B).

FOXO3a Activation Inhibits Neointimal Hyperplasia, Which Was Reversed by CYR61 Replenishment

Morphometric analysis at 14 days after balloon injury in rat carotid artery revealed that FOXO3a activation resulted in a significantly reduced neointimal area (0.06 ± 0.02 versus 0.20 ± 0.07 mm², $n=12$, $P < 0.01$), whereas there was no significant difference in medial area (0.17 ± 0.03 versus 0.16 ± 0.03 mm², $n=12$, $P=0.27$) compared with the control group (supplemental Figure IC). As a result, FOXO3a activation led to a significantly lower intima-to-media ratio (1.26 ± 0.42 versus 0.41 ± 0.20 , $P < 0.01$) and a greater lumen area (0.25 ± 0.08 versus 0.31 ± 0.06 mm², $P < 0.01$) compared with the control (Figure 6A, middle).

Conversely, when Ad-CYR61 was cotransfected with Ad-TM-FOXO3a, reduction in neointimal hyperplasia was significantly reversed near to the control level, suggesting CYR61 suppression contributed to reduction in neointimal hyperplasia by FOXO3a activation (Figure 6A, right).

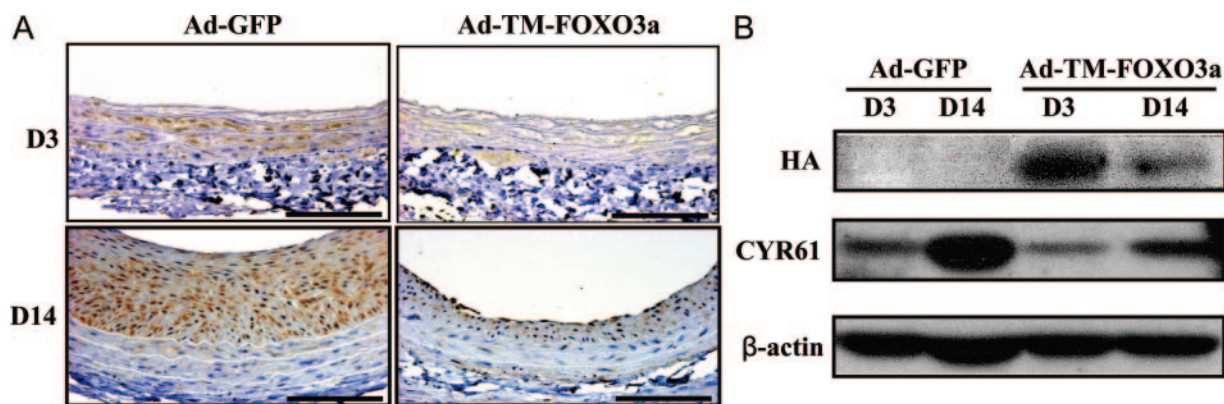


Figure 5. FOXO3a blocks CYR61 expression after balloon injury in rat carotid artery. A, Immunohistochemical staining showing increased expression of CYR61 at days 3 and 14 after balloon injury (left). The induction of CYR61 was blocked by FOXO3a-TM gene transfer both at day 3 and day 14 (right). B, Immunoblot confirming efficient delivery of FOXO3a-TM transgene (top). The induction of CYR61 after balloon injury was significantly decreased by FOXO3a-TM gene transfer both at day 3 and day 14 compared with control vessels (middle).

Then, we evaluated the role of CYR61 suppression by FOXO3a activation in apoptosis after balloon injury. TUNEL staining was performed at 3 and 14 days after balloon injury. Although only a few TUNEL-positive cells were observed at 14 days (data not shown), enough TUNEL-positive cells for comparison were observed at 3 days (Figure 6B). The apoptotic activity of VSMCs was higher in TM-FOXO3a-transfected group than the control group (33.0 ± 9.7 versus 66.0 ± 10.7 , $P < 0.05$; Figure 6B, middle). However, CYR61 replenishment did not exert significant effect on apoptosis (54.8 ± 16.7 , $P = 0.20$; Figure 6B, right).

Next, we evaluated the contributing role of CYR61 suppression in VSMC proliferation by PCNA staining. PCNA-positive cells were markedly reduced in TM-FOXO3a-transduced group 14 days after balloon injury (Figure 6C, middle). The proliferative index (calculated as the fraction of positive cells among total nucleated cells) was significantly lower in the Ad-TM-FOXO3a group compared with the control (15.0 ± 3.9 versus 36.2 ± 5.9 , $P < 0.05$), whereas CYR61 replenishment significantly reversed it (28.3 ± 7.5 , $P < 0.01$ compared with Ad-TM-FOXO3a group) (Figure 6C, right), suggesting that CYR61 suppression significantly contributed to reduced VSMC proliferation by FOXO3a activation.

Discussion

The present study demonstrates that FOXO3a is a negative transcriptional regulator of CYR61. FOXO3a activation significantly suppressed either serum- or angiotensin II-induced CYR61 expression in VSMCs. CYR61 suppression contributed to the effect of FOXO3a on reduction of VSMC proliferation and migration, resulting in reduction of neointimal hyperplasia. These data suggest that regulation of CYR61 may be a novel mechanism by which FOXO3a controls the vascular proliferative response to stimuli.

VSMC proliferation and migration are characteristic features of various vascular diseases, such as atherosclerosis and neointimal hyperplasia after vascular intervention. 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 cancer cell lines,^{24,25} fibro-

blasts,¹⁰ endothelial cells,¹⁵ and VSMCs.²⁶ In VSMCs, we previously showed that FOXO3a activation led to apoptosis and cell cycle arrest, thus inhibiting neointimal hyperplasia via p27^{Kip1} upregulation.¹¹ Although p27^{Kip1} upregulation was suggested to contribute to cell cycle arrest by forkhead transcription factors in the various cell lines mentioned above, it is unlikely that this is the only mechanism behind the actions of forkhead transcription factors. Considering the existence of many downstream molecules of forkhead transcription factor, it is more likely that other mechanisms may exist.

CYR61 is observed in VSMCs of arterial walls during embryonic development²⁷ and in arteriosclerotic arteries.⁷ CYR61 was reported to be associated with tissue responses to injury, such as bone fracture repair,²⁸ liver regeneration,²⁹ and granulation tissue formation during wound healing.³⁰ In addition, CYR61 was found to be highly expressed in the neointima of the balloon injured artery in rats³¹ and in primates.⁵ All of these findings suggest that CYR61 could be a potential target in neointimal hyperplasia. Despite several potential binding motifs of transcription factors, such as nuclear factor κ B, cAMP response element-binding protein/JUN, or activator protein-1 in the CYR61 promoter region,¹⁸ and several physical or chemical stimuli reported to induce CYR61 expression,³² the upstream signaling pathways of CYR61, apart from c-Jun N-terminal kinase¹⁴ and early growth response-1,³³ have not been discovered yet. Because CYR61 was found to have forkhead binding motif in its promoter region, we investigated the role of FOXO3a in CYR61 expression and found that FOXO3a is a negative transcriptional regulator of CYR61. Because, forkhead transcription factors are mainly regulated by Akt,¹⁰ we performed experiments evaluating CYR61 expression in the presence of dominant negative Akt to clarify the role of Akt in CYR61 expression (supplemental Figure ID). Induction of CYR61 expression by serum or angiotensin II was significantly blocked by dominant-negative Akt gene transduction, suggesting that Akt mediates CYR61 expression in response to growth factors. However, the inhibitory effect of dominant-negative Akt on CYR61 expression was completely abolished

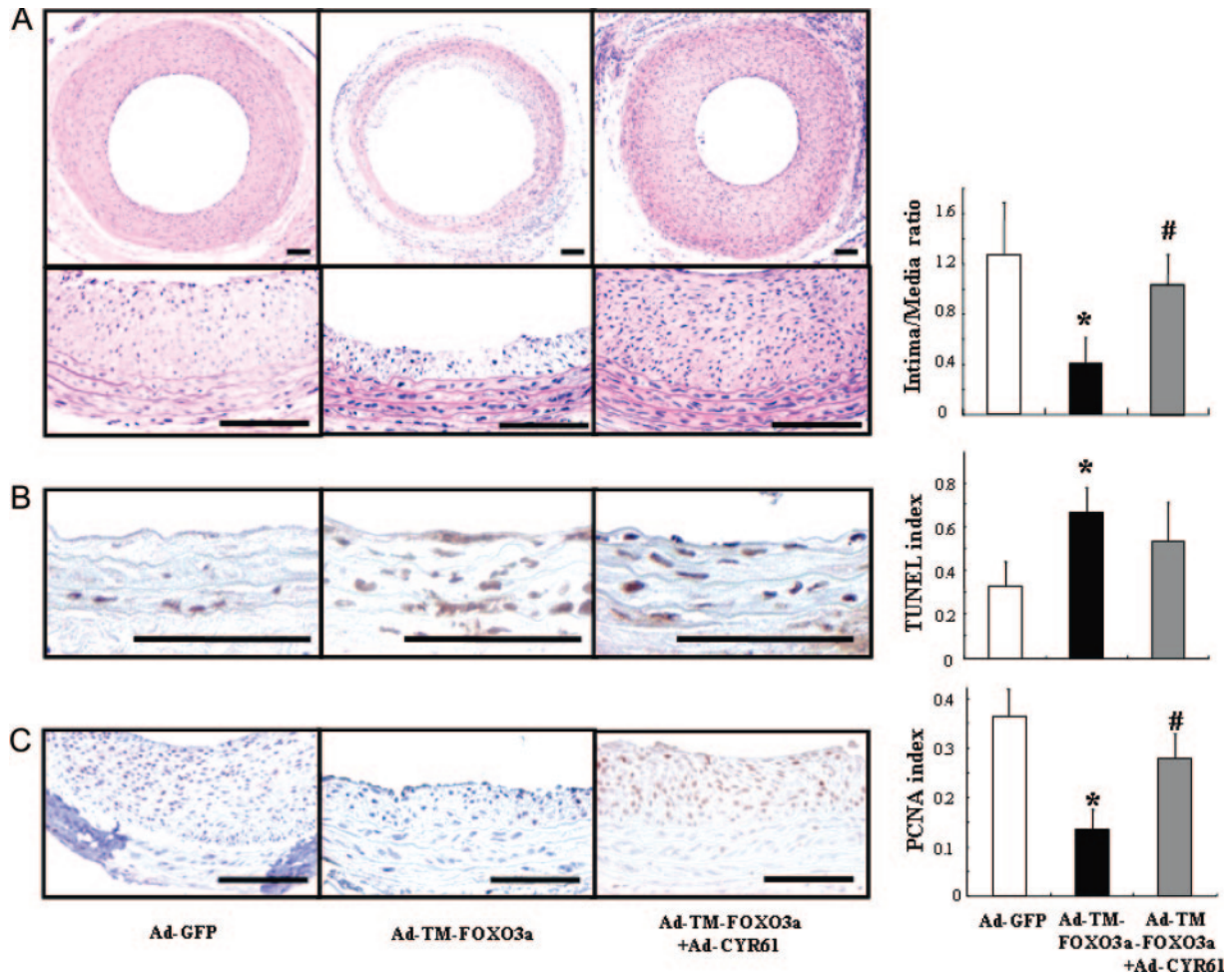


Figure 6. FOXO3a activation inhibits neointimal hyperplasia, which was reversed by CYR61 replenishment. A, Hematoxylin and eosin staining of arteries at 14 days after balloon injury and gene delivery. FOXO3a activation resulted in a significantly reduced neointimal area. This effect was near completely reversed by cotransfection of Ad-CYR61 with Ad-TM-FOXO3a. Magnification, $\times 100$ (top) and $\times 400$ (bottom). Bar graph illustrates intima/media ratio at 14 days after gene delivery. * $P < 0.05$ compared with the Ad-GFP, # $P < 0.05$ compared with the Ad-TM-FOXO3a ($n = 12$). B, TUNEL staining to detect apoptotic cells at day 3. Magnification, $\times 400$. Bar graph illustrates TUNEL index. * $P < 0.05$ compared with the Ad-GFP ($n = 6$). C, Immunohistochemical staining against PCNA at days 14. Magnification, $\times 200$. Bar graph illustrates PCNA index, * $P < 0.05$ compared with the Ad-GFP, # $P < 0.05$ compared with Ad-TM-FOXO3a ($n = 6$).

by cotransfection of adenovirus containing dominant-negative FOXO3a construct, indicating that the mechanism of dominant-negative Akt to blocking CYR61 induction is through FOXO3a activation and that Akt indirectly regulates CYR61 via inhibition of FOXO3a. Thus, Akt-FOXO3a pathway is the upstream regulator (Akt is positive, whereas FOXO3a is negative) of CYR61 expression. To the best of our knowledge, this is the first report to link the regulation of CYR61 expression with the Akt/FOXO3a axis. We think that this regulation is specific for CYR61 among various CCN family members, because we observed that connective tissue growth factor did not have a FOXO binding element in its promoter region and that its expression was not affected by FOXO3a (data not shown).

Although Ad-CYR61-transfected VSMCs showed more proliferation than the control group in our experiments, CYR61 has not been reported to have an intrinsic mitogenic effect, only a synergistic effect, with other mitogenic growth factors in fibroblasts³⁴ and in endothelial cells.³⁵ However, our results did not seem discrepant with respect to previous

reports, because we evaluated cell proliferation in the presence of growth stimulating condition such as serum or balloon injury. Furthermore, consistent with the previous report observed in endothelial cells,⁶ CYR61 was found to increase VSMC migration, which might contribute to the process of neointimal hyperplasia observed in this report.

Finally, it is possible that 2 regulatory mechanisms in VSMCs by FOXO3a, p27^{Kip1} upregulation and CYR61 suppression, might actually be linked with each other. CYR61 is implicated to regulate p27^{Kip1} expression through integrins such as $\alpha_v\beta_3$ in breast cancer or hepatocellular carcinoma cells.^{29,36} And connective tissue growth factor, another CCN family member, was reported to reduce p27^{Kip1} expression.³⁷ Therefore, upregulation of p27^{Kip1} by FOXO3a activation might actually be a secondary finding of CYR61 suppression. However, because p27^{Kip1} also has a FOXO3a binding motif in its promoter region,⁹ thus being potentially regulated by FOXO3a at the transcription level, we cannot make any conclusions regarding the relation between the 2 downstream molecules of FOXO3a, p27^{Kip1} and CYR61, at this time.

In summary, FOXO3a is a novel negative regulator of CYR61. CYR61 suppression contributed to reduction of neointimal hyperplasia by FOXO3a activation through decreased proliferation and possibly through reduction in migration of VSMCs. These findings offer new insights into the role of FOXO3a in the regulation of CYR61 and CYR61-dependent response after vessel injury.

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Disclosures

None.

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Correction

In an article by Lee et al (*Circ Res.* 2007;100:372-380), the authors regrettably supplied an incorrectly loaded gel for tubulin for Figure 3 panel A. The corrected figure appears here.

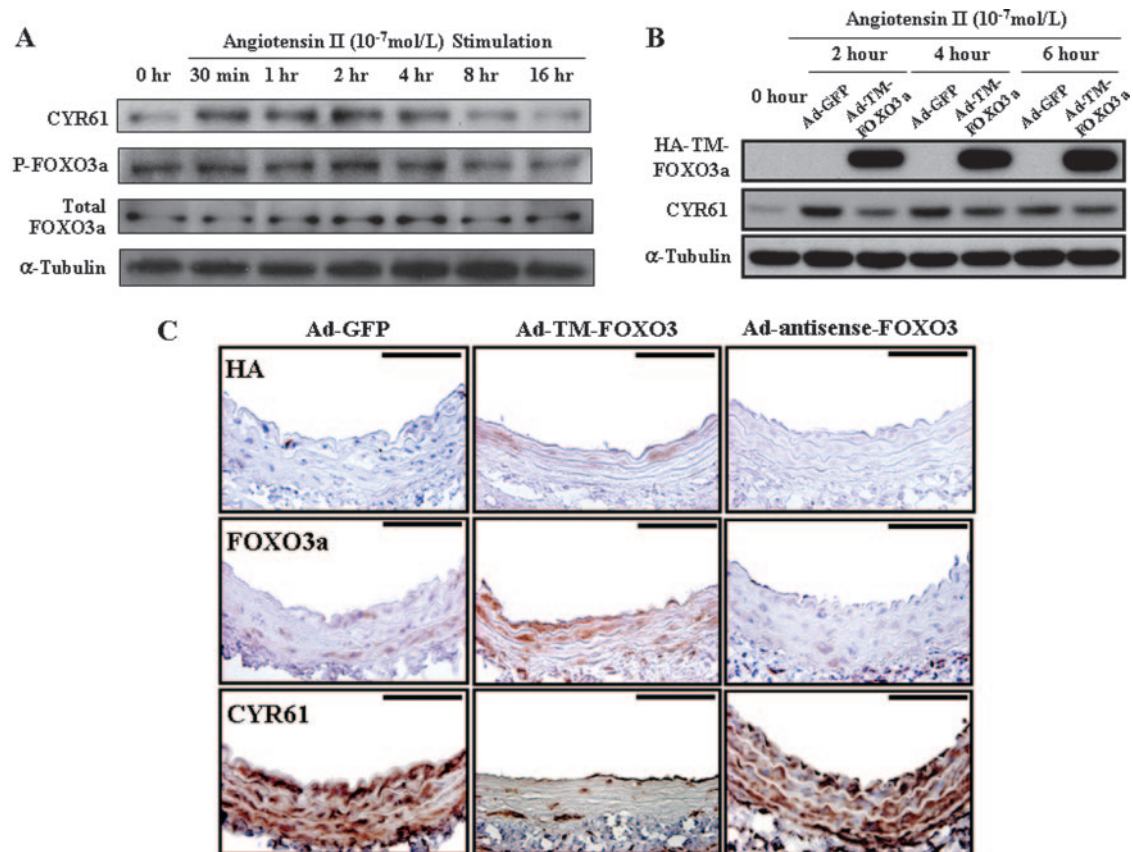


Figure 3. FOXO3a blocked angiotensin II-induced CYR61 expression. A, Serial immunoblot analysis of CYR61, phosphorylated (thus inactivated) native FOXO3a and total native FOXO3a after angiotensin II (10^{-7} mol/L) stimulation. B, Serial immunoblot analysis of CYR61 protein at 2, 4, and 6 hours after angiotensin II stimulation. C, Corresponding immunohistochemistry of FOXO3a and CYR61 2 hours after angiotensin II stimulation in rat aorta that were pretransfected with Ad-GFP (left), Ad-TM-FOXO3a (middle), and Ad-antisense-FOXO3a (right), respectively. Effective transfection of Ad-TM-FOXO3a and Ad-GFP to rat aorta was confirmed by staining for HA, whereas Ad-antisense-FOXO3a does not contain sequences for HA.

Supplemental Table I. FACS analysis of the cell cycle showing a typical profile of G0/G1 cell-cycle arrest in the TM-FOXO3a transfected group, in contrast to cell cycle propagation in the CYR61 group. Results are expressed as the mean \pm SE of a representative experiment, performed in triplicate wells (* $p < 0.05$, $n = 3$).

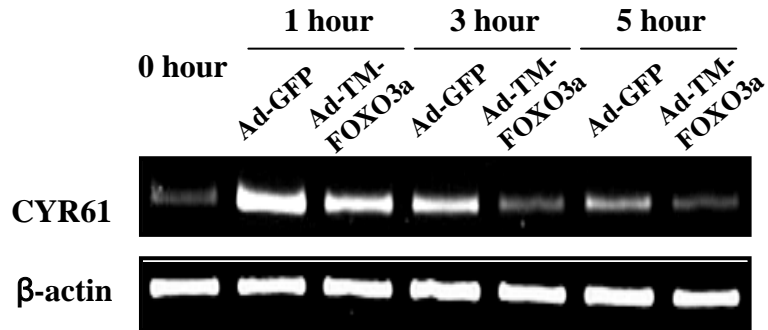
% of cells	Ad-GFP	Ad-CYR61	Ad-TM-FOXO3a	Ad-TM-FOXO3a + Ad-CYR61
G1	68.0 \pm 2.5	47.1 \pm 1.7*	71.2 \pm 1.0	68.0 \pm 2.5
S	24.0 \pm 0.9	40.0 \pm 2.1*	25.2 \pm 1.2	33.3 \pm 2.0**
G2 + M	8.0 \pm 0.7	13.3 \pm 0.9	3.6 \pm 0.5	11.8 \pm 1.0**

* $P < 0.05$ compared with the control, Ad-GFP group.

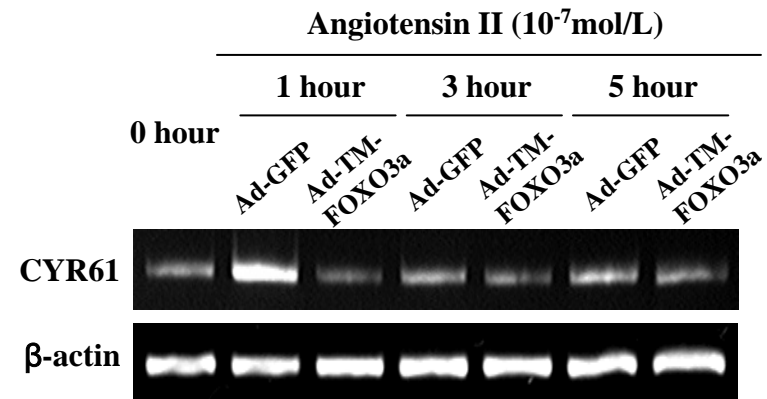
** $P < 0.05$ compared with Ad-TM-FOXO3a group.

Supplemental Figure I

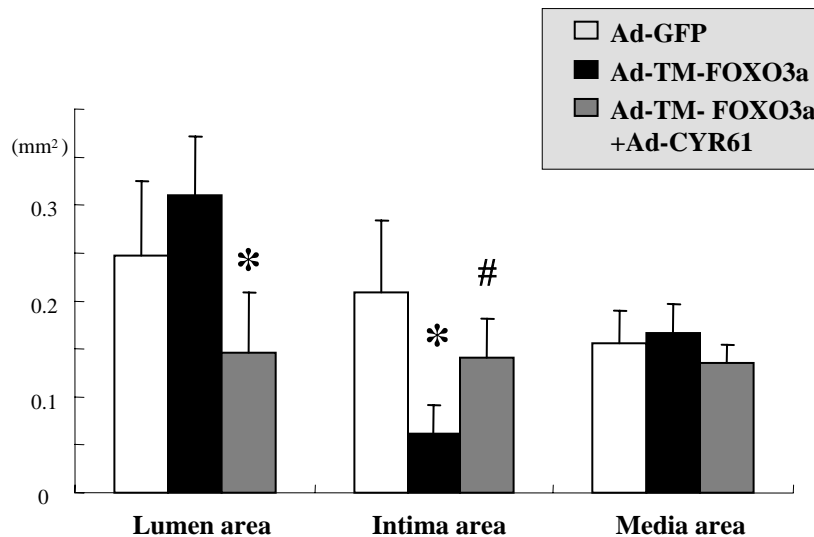
A. Serial RT-PCR analysis of CYR61 mRNA levels after 20% FBS stimulation.



B. Serial RT-PCR analysis of CYR61 mRNA levels after angiotensin II stimulation.



C. Morphometry at 14 days after gene delivery



D. Indirect regulation of CYR61 expression by Akt via FOXO3a inhibition

