Therapeutic Targeting of RNA Polymerase I With the Small-Molecule CX-5461 for Prevention of Arterial Injury–Induced Neointimal Hyperplasia

Qing Ye, Shu Pang, Wenjing Zhang, Xiaotong Guo, Jianli Wang, Yongtao Zhang, Yang Liu, Xiao Wu, Fan Jiang

Objective—RNA polymerase I (Pol I)–dependent rRNA synthesis is a determinant factor in ribosome biogenesis and thus cell proliferation. The importance of dysregulated Pol I activity in cardiovascular disease, however, has not been recognized. Here, we tested the hypothesis that specific inhibition of Pol I might prevent arterial injury–induced neointimal hyperplasia.

Approach and Results—CX-5461 is a novel selective Pol I inhibitor. Using this tool, we demonstrated that local inhibition of Pol I blocked balloon injury–induced neointima formation in rat carotid arteries in vivo. Neointimal development was associated with augmented rDNA transcriptional activity as evidenced by the increased phosphorylation of upstream binding factor-1. The beneficial effect of CX-5461 was mainly mediated by inducing G2/M cell cycle arrest of proliferating smooth muscle cells without obvious apoptosis. CX-5461 did not induce p53 stabilization but increased p53 phosphorylation and acetylation and activated the ataxia telangiectasia mutated/ataxia telangiectasia and Rad3-related (ATR) pathway. Inhibition of ATR, but not of ataxia telangiectasia mutated, abolished the cytostatic effect of CX-5461 and p53 phosphorylation. In addition, inhibition of p53 or knockdown of the p53 target GADD45 mimicked the effect of ATR inhibition. In vivo experiments showed that the levels of phospho-p53 and acetyl-p53, and activity of the ataxia telangiectasia mutated/ATR pathway were all augmented in CX-5461–treated vessels.

Conclusions—Pol I can be therapeutically targeted to inhibit the growth of neointima, supporting that Pol I is a novel biological target for preventing arterial restenosis. Mechanistically, Pol I inhibition elicited G2/M cell cycle arrest in smooth muscle cells via activation of the ATR-p53 axis.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:476-484. DOI: 10.1161/ATVBAHA.116.308401.)

Key Words: ataxia telangiectasia ■ cell cycle checkpoints ■ muscle, smooth ■ neointima ■ RNA polymerase I
18S, 5.8S, and 28S rRNAs; these rRNA transcripts are then assembled with ribosomal proteins to form the large (60S) and small (40S) subunits of the ribosome. Indeed, overactivation of the Pol I activity has been recognized as a hallmark of certain cancer cells. In eukaryotes, disruption of the normal nucleolar functions, such as inhibiting rDNA transcription with actinomycin D or inhibiting rRNA processing with 5-fluorouracil, induces a unique cellular stress response called nucleolar stress (or ribosomal stress), which results in stabilization of p53 protein by repressing p53 degradation by the E3 ubiquitin ligase MDM2.

CX-5461 is a novel, first-in-class selective Pol I inhibitor. Preclinical studies have shown that CX-5461 exhibits cytostatic or cytotoxic effects in both leukemic and solid cancer cells, although the mechanisms of these actions (either p53 dependent or p53 independent) remain controversial. The biological effects Pol I inhibition in vascular cells, however, are entirely unknown. Limited evidence has shown that proliferating VSMCs are associated with an increased rate of protein synthesis. In addition, it is clear that mammalian target of rapamycin is an important positive regulator of rDNA transcription, and the rate of rRNA synthesis is sensitive to rapamycin. Therefore, the potent cytostatic effect of rapamycin in VSMCs suggests the possibility that inhibiting rRNA synthesis may reduce the proliferating capability of the cell. Based on these lines of information, in the present study, we hypothesized that specific inhibition of Pol I with CX-5461 might be effective in blocking the development of proliferative neointimal lesions.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.
Results

CX-5461 Suppresses Balloon Injury–Induced Neointima Formation in the Carotid Artery

Given that there was no information about the effects of CX-5461 in the cardiovascular system, we started by characterizing the pharmacodynamic property of CX-5461 using VSMC proliferation as an end point. As shown in Figure 1A and 1B, CX-5461 inhibited proliferation of mouse aortic smooth muscle cell (MOVAS) in a concentration-dependent manner. The calculated EC50 value for this effect was 1.06 μmol/L. Based on these data, we selected 2 concentrations of CX-5461, namely, a sub-EC50 (0.7 μmol/L, low-dose) level and a supra-EC50 (14 μmol/L, high-dose) level, for following in vivo studies. We found that even at the low-dose, perivascular administration of CX-5461 effectively inhibited the neointima formation. CX-5461 at the higher concentration completely abolished the development of neointima (Figure 1C through 1E). Histological examination of the arterial cross sections revealed that CX-5461 had no obvious effects on the gross distribution and morphology of medial SMCs. To further verify whether CX-5461 had any proapoptotic effect in VSMCs, we treated MOVAS cells with increasing concentrations of CX-5461. Using both terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling and caspase3/7 activity assay, we demonstrated that CX-5461 even at the high dose did not induce significant apoptosis in VSMCs (Figure 1F and 1G).

Pol I Inhibition Induces G2/M Blockade in Proliferating Smooth Muscle Cells

To characterize the effects of CX-5461 on VSMC proliferation, we performed flow cytometry analysis in MOVAS cells; we found that CX-5461 induced prominent cell cycle block at the G2/M phase (Figure 2A). In a randomly cycling cell population, ≈3% of the cells showed positive nuclear labeling for Aurora B, a marker of the mitotic phase. In CX-5461–treated cells, no Aurora B–positive cells could be detected (Figure 2B). Conversely, CX-5461 significantly increased the number of cells with high expression of cyclin A (Figure 2C), a marker peaking in late S to G2 and diminishing rapidly in the prometaphase. In comparison, the proportion of cells expressing a high level of proliferating cell nuclear antigen, which was maximal in the S phase, was not significantly increased.

Figure 2. CX-5461 induces G2/M blockade in proliferating smooth muscle cells. A, Flow cytometry data showing concentration-dependent effects of CX-5461 on G2/M cell cycle arrest in mouse aortic smooth muscle cell line (MOVAS) cells. B, Effect of CX-5461 (14 μmol/L) on the number of Aurora B–high cells in randomly cycling MOVAS cells. Aurora B was highly expressed in cells in metaphase phase when chromosomes were aligned at the equatorial plate (arrow). C, Effects of CX-5461 on the proportion of cyclin A–high and proliferating cell nuclear antigen (PCNA)–high cells. D, CX-5461 treatment enlarged the size of nuclei. E, Effects of CX-5461 on cell cycle in rat primary vascular smooth muscle cells cultured in the absence and presence of platelet-derived growth factor (PDGF)-BB (20 ng/mL). Data are mean±SD. *P<0.05 vs control, unpaired t test, n=3 to 5. DAPI indicates 4’,6-diamidino-2-phenylindole.
changed by CX-5461 (Figure 2C). These data together indicate that CX-5461 induces accumulation of cells in the G2 phase. Consistently, we observed that CX-5461 treatment enlarged the size of nuclei (Figure 2D), a phenomenon indicating the induction of G2/M arrest.22 Since MOVAS is an immortalized cell line, it may not precisely reflect the behaviors of primary cells. Hence, we also tested the effects of CX-5461 in rat primary aortic SMCs cultured without and with the smooth muscle mitogen platelet-derived growth factor-BB. We found that CX-5461 similarly induced G2/M blockade in platelet-derived growth factor–stimulated primary VSMCs (Figure 2E). However, CX-5461 showed no significant effect in unstimulated cells (Figure 2E), indicating that quiescent cells were more resistant to inhibition of rDNA transcription. Hence, the following experiments with primary cells were performed in the presence of platelet-derived growth factor.

To further corroborate the cytostatic effect of CX-5461 in vivo, we did immunostaining for proliferating cell nuclear antigen, cyclin A, and Aurora B in arterial cross sections. We demonstrated that these cell cycle markers were abundantly expressed in the proliferating neointimal cells; CX-5461 diminished the neointimal cells expressing these markers (Figure 3). Aurora B–positive cells were undetectable in the tunica media in either normal or injured arteries. In comparison, basal levels of proliferating cell nuclear antigen and cyclin A were observed in the medial layer of normal arteries. Because nuclear localization of cyclin A was essential for its cell cycle regulatory activity,23 we counted cells with high cyclin A expression which overlapped the nuclei. Balloon injury somehow decreased the prevalence of cyclin A–high cells in the media, probably because of concomitant emigration of the activated smooth muscle, whereas CX-5461 significantly increased the number of cyclin A–high cells in the media (Figure 3). In contrast, the prevalence of proliferating cell nuclear antigen–high cells in the media was not significantly changed by CX-5461 (Figure 3). These data, together with the in vitro results in Figure 2, indicated that CX-5461 induced a cell cycle arrest at the G2 phase in vivo.

**CX-5461 Does Not Induce p53 Stabilization but Increases p53 Phosphorylation**

To clarify whether the cell cycle arrest induced by Pol I inhibition was related to induction of nucleolar stress, we monitored subnuclear localization of nucleophosmin/B23 in primary VSMCs. Although CX-5461 at 700 nmol/L did not significantly alter nucleophosmin/B23 localization in the nucleoli, CX-5461 at 14 μmol/L induced nucleophosmin/B23 relocation to the nucleoplasm (Figure 4A), indicating the presence of nucleolar stress.24,25 However, we could not detect p53 protein stabilization in CX-5461–treated cells with either Western blotting or immunofluorescence (Figure 4B), suggesting that the effects of CX-5461 could not be fully explained by induction of the canonical nucleolar stress pathway. Interestingly, we found that CX-5461 treatment induced prominent p53 phosphorylation in primary VSMCs (Figure 4C). Because phosphorylation increases p53 activity, we determined whether p53 had a crucial role in CX-5461–induced effects on cell proliferation by pretreating primary VSMCs with the selective p53 inhibitor pifithrin-α; we demonstrated that the G2/M blocking effect of CX-5461 was diminished by pifithrin-α (Figure 4D). Moreover, we demonstrated that CX-5461 significantly increased the expression levels of p53 target genes p21 CIP/WAF1 and GADD45 (Figure 4E). Whereas p21 CIP/WAF1 had a critical role primarily in regulating G1/S transition, GADD45 had been shown to have an important role in mediating G2/M blockage.26,27 We treated MOVAS cells with adenoviruses expressing a GADD45 shRNA. GADD45 shRNA alone significantly increased the basal rate of cell proliferation (Figure 4F). In the presence of GADD45 shRNA, CX-5461–induced cytostatic effect was blunted (Figure 4F). However, we were unable to repeat the flow cytometry analysis in adenovirus-transfected cells because the presence of viral particles impeded precise detection of the host genomic DNA content.

**Pol I Inhibition Activates the Ataxia Telangiectasia Mutated/Ataxia Telangiectasia and Rad3-Related Pathway in Smooth Muscle Cells**

It is well established that p53 phosphorylation at Ser15 is controlled by the ataxia telangiectasia mutated (ATM)/ataxia telangiectasia and Rad3-related (ATR) pathway,28 which also has a pivotal role in regulating G2/M transition.29 To elucidate whether activation of the ATM/ATR pathway was involved in the effects of Pol I inhibition, we performed Western blotting and immunofluorescence analyses in primary VSMCs using an antibody recognizing the phospho-S/T*Q motif of ATM/ATR substrates. As shown in Figure 5A and 5B, CX-5461...
increased the phosphorylation levels of ATM/ATR substrates. Next, we pretreated VSMCs with the selective ATM inhibitor KU-55933 and the ATR inhibitor VE-821. We found that VE-821, but not KU-55933, abolished the cell cycle blocking effect of CX-5461 and CX-5461–induced p53 phosphorylation (Figure 5C and 5D). To further corroborate the roles of p53 and ATR in CX-5461–induced cytostatic effect, we repeated some of the above experiments in MOV AS cells. Similar to the results obtained in primary cells, we showed that CX-5461 increased p53 phosphorylation and ATM/ATR activation. Moreover, pifithrin-α and VE-821 attenuated the cytostatic effect of CX-5461 in MOVAS cells (Figure I in the online-only Data Supplement).

**Pol I Inhibition Induces p53 Acetylation**

Evidence suggests that p53 phosphorylation can coordinate other post-translational modifications such as acetylation, and multiple post-translational modifications of p53 seem to be more functionally important than individual modifications. Therefore, we also tested the effect of CX-5461 on the level of p53 acetylation in primary VSMCs. We also confirmed the effect of CX-5461 on p53 acetylation in MOVAS cells (Figure I in the online-only Data Supplement).

**CX-5461 Increases p53 Post-Translational Modifications and ATM/ATR Activation In Vivo**

To determine whether our findings in cultured VSMCs were relevant to the actions of CX-5461 in vivo, we performed immunohistochemistry experiments in arterial cross sections. We demonstrated that phosphorylation of upstream binding factor-1 (UBF1, a nucleolar transcription factor essential for rDNA transcription) on S484 was significantly increased in the neointimal area and the tunica media of injured arteries (Figure 6A), indicating an enhancement in the process of rRNA synthesis. Using antibodies to phospho-p53, acetyl-p53, and phospho-S/T*Q, we showed that p53 phosphorylation and acetylation and activation of the ATM/ATR pathway were all significantly augmented in the medial layer of CX-5461–treated arteries (Figure 6B). The levels of these markers were also highly expressed in the neointima in untreated arteries.

We also performed Western blot experiments and confirmed that, as compared with sham tissues, phospho-p53, acetyl-p53, and phospho-S/T*Q were all increased in
CX-5461 (high-dose)–treated vessels, which contained virtually no neointima (Figure 6C). The higher levels of these markers in balloon-injured vessels were likely to be because of the increased expressions in the neointima, as indicated by immunohistochemistry staining (Figure 6B). Moreover, using quantitative polymerase chain reaction, we confirmed that the in vivo expression levels of p21CIP/WAF1 and GADD45 were significantly increased in high-dose CX-5461–treated vessels when compared with sham (Figure II in the online-only Data Supplement). The levels of p21CIP/WAF1 and GADD45 were also higher in balloon-injured vessels comparing to sham, although these changes did not reach statistical significance (Figure II in the online-only Data Supplement).

Effects of CX-5461 on the Process of Re-Endothelialization

One major drawback of the current antiproliferation drugs is that they concurrently delay the process of re-endothelialization. To address this question, we examined the effects of CX-5461 on endothelial cells both in vitro and in vivo. In cultured human umbilical vein endothelial cells, CX-5461 at 0.7 and 14 μmol/L significantly reduced cell proliferation (Figure IIIA in the online-only Data Supplement). CX-5461 at the low dose did not significantly delay the endothelial recovery process, whereas at the high dose, it inhibited the rate of re-endothelialization by ≈35% (Figure III in the online-only Data Supplement). The currently used antirestenotic drugs such as rapamycin exhibited strong inhibitory effects on both migration and proliferation of endothelial cells.32 It is argued that although CX-5461 is not totally void of inhibitory effects on endothelial cell regrowth, the lack of effect of CX-5461 at the low dose on endothelial cell migration may represent an advantage over rapamycin derivatives.32

Discussion

In the present study, we have provided evidence showing that local treatment with the specific Pol I inhibitor CX-5461 can effectively prevent the development of neointima hyperplasia after balloon-induced arterial injury. Pol I–dependent rDNA transcription is a tightly regulated cellular process, which is essential for a sustained protein translational capacity required for rapid cell growth, and is sensitive to mitogenic stimuli and cellular stresses.10,11 In line with its fundamental role in maintaining cell growth, increased Pol I activity and resultant rDNA transcription are found in human cancers.8 For example, overactivation of Pol I has been shown to be indispensable for the survival of leukemic cancer cells, and importantly,16 in most tissues, nevertheless, Pol I–dependent transcription is regarded as a housekeeping process. The importance of dysregulated Pol I activity and rDNA transcription in cardiovascular disease has not been recognized. Our results indicate that the activity of the rDNA transcriptional machinery is augmented during the process of neointimal proliferation, as evidenced by the increased UBF phosphorylation.10 More importantly, we have demonstrated that Pol I can be therapeutically targeted in vivo to repress the growth of neointima,
in contrast to that observed in leukemic cells, indicating that Pol I inhibitor is not associated with increased cell apoptosis, supporting the notion that RNA Pol I is a novel biological target of rapamycin inhibitors, which induce prominent G1/S cell cycle arrest, with little effect on the G2/M transition, indicating involvement of different signaling mechanisms. Whereas it is suggested that mammalian target of rapamycin inhibitors elicit smooth muscle cytostasis primarily by upregulating expression of the cell cycle inhibitor p27^Kip1, our study reveals that Pol I inhibitor-induced cell cycle arrest is associated with activation of the ATM/ATR pathway, which is in line with the observations in cancer cells.

ATM and ATR kinases are key mediators of the DNA damage response in eukaryotic cells, which induce cell cycle arrest and facilitate DNA repair via various downstream targets. It is noted that ATM and ATR are not functionally redundant although some of the functions of these 2 kinases are partially overlapping. Our results strongly suggest that the ATR kinase is indispensable for the effects of CX-5461, whereas ATM seems to be unimportant. This finding is supported by a recent study in HeLa cells, showing that inhibiting rRNA synthesis with a low concentration of actinomycin D causes ATR-dependent G2 arrest, with no effect on traverse of the G1 and S phase. Mounting evidence has shown that the ATR kinase is activated by not only DNA damages or replication stress but also various cellular stressors including hypoxia, mechanical stress, and oxidative stress, without evidence of DNA breaks. Taken together, we suggest that ATM and ATR pathways may transduce distinct stress signals and lead to different cellular outcomes in VSMCs.

Currently, it is not clear how CX-5461 activates the ATM/ATR pathway. Based on the above findings, it is plausible that Pol I inhibitor–induced ATM/ATR activation and cell cycle arrest are not dependent on bona fide DNA damages. One possibility is that CX-5461 disrupts normal assembly of the rDNA transcriptional machinery, leading to local nucleolar chromatin conformational changes, such as chromatin condensation, which may be sufficient to trigger DNA damage response in the absence of DNA breaks. Nevertheless, this hypothesis remains to be confirmed by further experiments.

ATR kinase may regulate G2/M cell cycle arrest via 2 mechanisms, namely, phosphorylation of the downstream kinase CHK1 and phosphorylation and activation of the tumor suppressor p53. Phosphorylation of p53 can further orchestrate acetylation of the protein, leading to full activation of its transcriptional functions. The functional importance of p53 in mediating cell cycle arrest and growth inhibition in VSMCs has been well documented. In contrast, there is little information on the role of CHK1 in VSMCs. Interestingly, a recent study indicates that phosphorylation of CHK1 does not have a significant role in radiation-induced DNA damage response in stem cell–derived smooth muscle cells. Hence, in the present study, we focused on a possible involvement of p53 in CX-5461 effects in VSMCs. We have shown that p53 phosphorylation is increased in an ATR-dependent manner after CX-5461 treatment, whereas the level of p53 acetylation is also augmented concomitantly. Consistently, CX-5461 increases the expression of p53 target genes GADD45 and p21^CIP/WAF1. In addition, treatment with a specific p53 inhibitor or GADD45 gene silencing mimics the effect of ATR inhibitor on the actions of CX-5461. Taken together, these data strongly support the notion that RNA Pol I is a novel biological target for preventing arterial injury–induced restenosis.

Our data show that the neointimal suppressing effect of Pol I inhibitor is not associated with increased cell apoptosis, in contrast to that observed in leukemic cells, indicating that VSMCs, either in a proliferating or quiescent state, are more resistant to Pol I inhibition–induced stress. On the contrary, using both flow cytometry and immunofluorescence, we have demonstrated that the beneficial effect of CX-5461 is mainly achieved by inducing cell cycle arrest of proliferating VSMCs at the G2/M checkpoint. This cytostatic effect of CX-5461 is consistent with that reported by Negi and Brown in leukemic lymphoblastic cells although apoptosis is also induced by the drug in these cells. Obviously, the effect of Pol I inhibitor on VSMC proliferation is distinct from those of mammalian
suggest that the ATR-p53 axis has a pivotal role in mediating the cell cycle blocking effect of CX-5461 in VSMCs. This notion is further supported by the in vivo experiments, showing that the levels of phospho-p53, acetyl-p53, and phospho-S/T*Q are all augmented in CX-5461–treated vessels. Unexpectedly, we cannot detect p53 protein stabilization in CX-5461–treated VSMCs as that observed in the leukemic cells38; this phenomenon is not consistent with the conventional nuclear stress paradigm, indicating potential cell type–specific variations.

It should be noted, however, our results cannot totally exclude the involvement of p53-independent pathways (such as CHK1) downstream of ATR kinase in CX-5461–induced effects, given the multifaceted functions of ATR and ATM pathways.29,36,51 Moreover, we cannot exclude that CX-5461 also interrupts the cell cycle at the G1/S interface because both ATR and p53 can induce either G1/S arrest or S phase delay.29,36,44 Notwithstanding, all of these alternative pathways will eventually converge to inhibit smooth muscle cell proliferation, which is the fundamental biological mechanism for preventing neointima formation.

In summary, our study provides the first evidence suggesting that Pol I can be therapeutically targeted in vivo to repress the growth of neointima, supporting the notion that Pol I is a novel biological target for preventing arterial injury–induced restenosis. Mechanistically, the beneficial effect of Pol I inhibitor is mainly through inducing cell cycle arrest of proliferating VSMCs at the G2/M checkpoint, whereas the ATR-p53 axis has a pivotal role in mediating the cell cycle blocking effect of Pol I inhibitor.

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Disclosures
None.

References

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Supplemental Figure I. Roles of p53 and ATR in CX-5461-induced cytostatic effect in MOVAS cells. (A) Western blots and relative densitometry data showing the effects of CX-5461 (0.7 µM) on p53 phosphorylation and acetylation. (B) Effects of CX-5461 (0.7 µM) on phosphorylation of the ATM/ATR substrate motif S/T*Q. (C) The p53 inhibitor pifithrin-α (Pif, 10 µM) and VE-821 (400 nM) suppressed the cytostatic effect of CX-5461 measured by flow cytometry. Data are mean ± SD. * P < 0.05 versus control, unpaired t-test, n = 3-4.
**Supplemental Figure II.** Real-time PCR results showing the expression levels of p21\textsuperscript{CIP/WAF1} and GADD45 in sham and balloon (Bal)-injured vessels without and with high dose CX-5461 treatment. Data are mean ± SD. * $P < 0.05$, one-way ANOVA, $n = 5-6$. 

![Graph showing p21 and GADD45 mRNA expression levels](image-url)
Supplemental Figure III. Effects of CX-5461 on endothelial cell regeneration. (A) Effects of CX-5461 on proliferation of human umbilical vein endothelial cells measured with a colorimetric assay. (B) Effects of CX-5461 on cell migration assessed by monolayer wound healing assay. (C) Effects of CX-5461 on cell migration assessed by Boyden chamber assay. (D) Effects of CX-5461 at different doses on re-endothelialization of the denuded area in balloon-injured arteries in vivo. Data are presented as mean ± SD. * P < 0.05, one-way ANOVA, n = 3-6.
Materials and methods

Reagents

CX-5461, pifithrin-α, VE-821 and KU-55933 were purchased from Selleck Chemicals (Houston, TX, USA). Platelet-derived growth factor (PDGF)-BB was from R&D Systems (Minneapolis, MN, USA). Primary antibodies against Aurora B (#ab3609), NPM/B23 (#ab10530), acetyl-p53 (K381/382) (#ab61241), p21CIP/WAF1 (#ab7960), phospho-UBF1 (S484) (#ab182583), and proliferating cell nuclear antigen (PCNA) (#ab18197) were from Abcam (Cambridge, UK). Antibodies against p53 (#2524), acetyl-p53 (K381/382) (#2525), phospho-p53 (Ser15) (#9284), GADD45 (#4632), and phospho-ATM/ATR substrates (S*Q) antibody mix (#9607) were from Cell Signaling (Beverley, MA, USA). Antibodies against cyclin A (#18202-1-AP) and p53 (#10442-1-AP) were from Proteintech (Rosemont, IL, USA). Purified adenoviral vectors expressing GADD45-targeting shRNA (sequence TTCTCCGAACGTGCTACGTTTC) were obtained from Genepharma (Shanghai, China).

Animal experiments

All animal experiments were approved by the Qilu Hospital Animal Ethics Committee, and performed in accordance with the ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines. Male Wistar rats weighing ~250-300 g were purchased from Vital River Laboratories (Beijing, China) and maintained on standard chow diet and water ad libitum. Carotid artery balloon injury was performed according to the protocol provided by Tulis. Briefly, rats were anesthetized using pentobarbital sodium (60 mg/kg, i.p.). The entire length of the left common carotid artery and the external carotid branch were exposed. The blood flow was stopped by clamping the common carotid artery. An arteriotomy incision was made in the external carotid branch distally, and a 1.25-mm balloon catheter (Medtronic, Minneapolis, MN, USA) was introduced into the common carotid artery. The balloon was slowly inflated to a pre-determined pressure of 3 atm, and the injury was induced by withdrawing the catheter with rotation. The catheter was deflated and the process was then repeated for additional 2 times. After surgery, a dose of meloxicam (1 mg/kg, s.c.) was given for analgesia. For peri-vascular treatment, CX-5461 was first dissolved in DMSO and mixed with 25% Pluronic F-127 gel (from Sigma-Aldrich, Shanghai, China) to final concentrations of 0.7 (low dose) or 14 µM (high dose), and stored at 4°C. Equivalent amount of DMSO was used as vehicle control. After balloon injury, 200 µL of the gel solution was applied over the artery and allowed to solidify.

The process of re-endothelialization was evaluated in vivo according to Morita and co-workers. Briefly, 30 minutes before euthanasia, rats were injected intravenously via the tail vein with 0.5 mL of 0.5% Evans blue dye, which specifically stains denuded areas of the arterial wall. Arteries were fixation in 100% methanol and opened longitudinally. Endothelialization was quantified by calculating the percentage of non-stained area to the entire injured area. This was performed in a blind manner.

Histology and morphometry analysis

Carotid arteries were collected 14 days after injury, fixed in 4% paraformaldehyde and embedded in paraffin. Cross-sections of 4-µm thickness were cut and stained with hematoxylin and eosin. Sections were also stained for elastic tissues with Verhoeff-van Gieson staining as used previously. Morphometric measurements of the total area of intima and the intima to media area ratio were performed in a blind manner using Image-Pro Plus 5.0 software from Media Cybernetics (Bethesda, Md., USA) as performed in our previous studies.
Cell culture
The mouse aortic smooth muscle cell line (MOVAS) was obtained from ATCC (CRL-2797), and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Primary VSMCs were isolated from rat aorta by digesting with an enzyme mixture containing collagenase I (1 mg ml\(^{-1}\)), elastase (0.5 mg ml\(^{-1}\)) and trypsin (1.25 mg ml\(^{-1}\)), as we used previously. Smooth muscle cells were cultured in DMEM supplemented with 10% FBS. Cells below passage 8 were used for experimentation. Human umbilical vein endothelial cells were cultured in complete ECM medium (Catalogue #1001, ScienCell, Carlsbad, CA, USA) supplemented with 5% FBS and the Endothelial Cell Growth Supplement as described.

Cell cycle analysis
Cell cycle was analyzed using flow cytometry (FACSCalibur from BD Biosciences, Mountain View, CA, USA). Cells were detached with trypsin and fixed overnight in cold ethanol. Propidium iodide staining was performed using a kit from Abcam (ab139418) according to the manufacturer’s instructions.

Cell proliferation and migration assays
Cell proliferation was assessed with a colorimetric tetrazolium-based assay using CellTiter 96 Aqueous kit (from Promega, Madison, WI, USA) according to the manufacturer’s direction. Cell migration was assessed using Boyden chamber as described previously. The membrane was fixed and cells counted under a light microscope 6 hours after seeding. Monolayer wound healing assay was performed as described.

Immunohistochemistry
Tissue sections were deparaffinized, and heated in a citrate buffer (pH 6.0) in a microwave oven for 15 min at high power to retrieve antigens. Sections were then treated with 3% (v/v) hydrogen peroxide at 37°C for 10 min to quench the endogenous peroxidase activity, and blocked with normal horse serum at 37°C for 1 h. Tissues were incubated with various primary antibodies at 4°C overnight followed by corresponding biotin-conjugated secondary antibodies at 37°C for 60 min. Slides were developed using a Vectastain Elite ABC Kit (from Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine (DAB) substrate. Negative control slides were included in all staining experiments by replacing the primary antibody with non-immune IgG to exclude the presence of non-specific background. To quantify the immunoreactivity, if the antigen expression level in individual cells can be distinguished in a binominal manner, the percentage of cells with high expression was calculated; if the antigen expression appeared to be a continuum, an arbitrary score of 1 to 5 (representing weakest to strongest expression) was assigned to each section by a blind reviewer, and the averaged score was presented.

Immunofluorescence
Cells cultured on Lab-Tek II chamber slides (Thermo Scientific, Waltham, MA, USA) were fixed in cold methanol. Cells were incubated with diluted primary antibodies overnight and then with Alexa Fluor-594 or -488-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at room temperature for 2 hr, and counter stained with DAPI for 15 min. Stained cells were analyzed using a confocal microscope (Model LSM710, Zeiss, Jena, Germany).

Detection of cell apoptosis
Cell apoptosis was detected using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and caspase 3/7 activity assay. TUNEL was performed using an ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (from Merck Millipore) following the manufacturer’s protocol. For measuring caspase activity, cells seeded in 96-well plates were incubated with the Caspase-Glo 3/7 substrate reagent (Promega, Madison, WI, USA) at 37°C for 30 min. The samples were transferred to a white-walled plate and the luminescence signals were detected using a Varioskan Flash plate reader (Thermo Scientific).

**Western blot**

Cells were lysed in a buffer containing 50 mM Tris, pH 7.5, 2 mM EDTA, 100 mM NaCl, 50 mM NaF, 1% Triton X-100, 1 mM Na3VO4 and 40 mM β-glycerol phosphate, and the protease inhibitor cocktail (Roche, Mannheim, Germany). Protein samples were separated by SDS-PAGE and transferred to PVDF membranes. The membrane was blocked with 5% nonfat milk, incubated with specific primary antibodies overnight, followed by horseradish peroxidase-conjugated secondary antibodies. The membrane was developed with ECL Prime reagents (GE, Piscataway, NJ, USA) and detected with a LAS-4000 luminescent image analyzer (Fujifilm, Stamford, CT, USA). Band densitometry analysis was performed using Image-J software (NIH).

**Statistical analysis**

Data are presented as mean ± SD. Data analysis was performed with unpaired t-test or one-way ANOVA followed by post hoc Newman-Keuls test as appropriate. P < 0.05 was considered as statistically significant.

**References**