LincRNA-p21 Regulates Neointima Formation, Vascular Smooth Muscle Cell Proliferation, Apoptosis, and Atherosclerosis by Enhancing p53 Activity

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Background—Long noncoding RNAs (lncRNAs) have recently been implicated in many biological processes and diseases. Atherosclerosis is a major risk factor for cardiovascular disease. However, the functional role of lncRNAs in atherosclerosis is largely unknown.

Methods and Results—We identified lincRNA-p21 as a key regulator of cell proliferation and apoptosis during atherosclerosis. The expression of lincRNA-p21 was dramatically downregulated in atherosclerotic plaques of ApoE−/− mice, an animal model for atherosclerosis. Through loss- and gain-of-function approaches, we showed that lincRNA-p21 represses cell proliferation and induces apoptosis in vascular smooth muscle cells and mouse mononuclear macrophage cells in vitro. Moreover, we found that inhibition of lincRNA-p21 results in neointimal hyperplasia in vivo in a carotid artery injury model. Genome-wide analysis revealed that lincRNA-p21 inhibition dysregulated many p53 targets. Furthermore, lincRNA-p21, a transcriptional target of p53, feeds back to enhance p53 transcriptional activity, at least in part, via binding to mouse double minute 2 (MDM2), an E3 ubiquitin-protein ligase. The association of lincRNA-p21 and MDM2 releases MDM2 repression of p53, enabling p53 to interact with p300 and to bind to the promoters/enhancers of its target genes. Finally, we show that lincRNA-p21 expression is decreased in patients with coronary artery disease.

Conclusions—Our studies identify lincRNA-p21 as a novel regulator of cell proliferation and apoptosis and suggest that this lncRNA could serve as a therapeutic target to treat atherosclerosis and related cardiovascular disorders. (Circulation. 2014;130:1452-1465.)

Key Words: apoptosis • atherosclerosis • cell proliferation • MDM2 protein • RNA, long noncoding • tumor suppressor protein p53

Atherosclerosis is one of the most common vascular disorders. Proliferation of vascular smooth muscle cells (VSMCs) and the formation of neointima dominate atherosclerotic lesion development. p53, an essential molecule in cell cycle and apoptosis control, also plays a central role in atherosclerosis.1-3 Inactivation of p53 stimulates the development of atherosclerosis.4-6 A complex network that includes p300 and mouse double minute 2 (MDM2) regulates p53. p300 is an acetyltransferase that acetylates p53 to enhance its activity.7-9 MDM2, an E3 ubiquitin-protein ligase, degrades p53 via the ubiquitin-proteasome pathway.10,11 MDM2 also blocks P300 interaction with p53, thereby inhibiting p53 acetylation and decreasing its activity.12-14 Intriguingly, whereas MDM2 is a key regulator of the fate and activity of p53, the transcription of MDM2 itself is under the control of p53, establishing a p53/MDM2 negative feedback loop.

Clinical Perspective on p 1465

More than 90% of the human genome is transcribed.15-17 Whereas protein-coding genes account for <2% of the human genome, noncoding RNAs (ncRNAs) are important components of the mammalian transcriptome.18,19 There are several interactions that govern ncRNA function.
classes of ncRNAs, including the well-known microRNAs, which are ≈21 to 23 nucleotides long and have been proven to play a key role in the regulation of gene expression and function in a variety of biological and pathological processes. Long noncoding RNAs (lncRNAs), also known as long intergenic noncoding RNAs (lincRNAs), constitute...
another class of ncRNAs. Defined as noncoding transcripts longer than 200 nucleotides, at least a subset of IncRNAs are likely to have biological activity.24–26 Numerous studies have already shown the involvement of IncRNAs in cancer development.27–29 However, the role of IncRNAs in cardiovascular system is less understood.24 A recent study discovered Braveheart, a heart-associated murine IncRNA, and demonstrated that Braveheart is essential for the maintenance of the fate of cardiomyocytes.31 Previous studies linked ANRIL (CDKN2B-AS), an IncRNA located at human chromosome 9p21.3, to increased coronary artery disease risk.32 However, the molecular nature of how this IncRNA regulates atherosclerosis process is unclear.

LincRNA-p21 was initially identified as a direct transcriptional target of p53.33 LincRNA-p21 appears to function as a component of the p53 pathway, at least in part, by physically interacting with a p53 repressive complex to downregulate many p53 target genes.33 LincRNA-p21 also acts as a suppressor of translation by directly associating with target mRNAs.34 Despite these studies, the biological function of lincRNA-p21 remains elusive.

In this study, we examined the functional role of lincRNA-p21 in the pathogenesis of atherosclerosis. We showed that the expression level of lincRNA-p21 was lower in atherosclerotic plaques of the ApoE−/− mice. Importantly, we found that inhibition of lincRNA-p21 increased cell proliferation and...
neointima formation in injured carotid arteries. Among the genes dysregulated by lincRNA-p21 inhibition was MDM2. We show that MDM2 interacts with lincRNA-p21 to relieve its repression of p53. Our study therefore uncovered a critical function of lincRNA-p21 in atherosclerosis.

**Methods**

**Cell Culture, Transfection, Cellular Proliferation, and Apoptosis Analysis**

The human VSMC line HA-VSMC and mouse mononuclear macrophage cell line RAW264.7 were purchased from ATCC and cultured, following the manufacturer’s instructions. Cells were transfected with Lipofectamine 2000.

Cells were seeded in 96-well plates, and cell proliferation was tested with the Cell Counting Kit-8 assay kit. Proliferating HA-VSMCs were quantified by Ki67 staining. For the cellular apoptosis assay, HA-VSMCs were seeded in 48-well plates, and terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assay was performed with the ApopTag Plus In Situ Apoptosis Fluorescein Detection Kit.

**RNA Isolation, Quantitative Reverse Transcription–Polymerase Chain Reaction Analysis, and Unbiased Gene Expression Profiling**

Total RNAs were isolated using TRIzol reagent. Reverse transcription (RT) and real-time quantitative polymerase chain reaction (PCR) were performed, following the manufacturer’s instructions. PCR primers are listed in Table I in the online-only Data Supplement. Unbiased genome-wide transcriptome profiling was performed by use of the HUGENE 2.0 ST array (Affymetrix), which interrogates a total of 40,716 Refseq transcripts. Array hybridization, signal detection, and data analysis were performed as described.

**RNA Immunoprecipitation Assay**

RNA immunoprecipitation (RIP) experiments were performed with the Magna RIP RNA-Binding Protein Immunoprecipitation Kit.
following the manufacturer’s instructions. Two independent MDM2 antibodies were used.

**Biotin RNA Pull-Down Assay, Deletion Mapping, and Bioinformatics**

RNA pull-down assay and deletion mapping were performed as described previously. Briefly, the pcDNA3.1-lincRNA-p21 plasmid was used as a template to synthesize biotinylated lincRNA-p21 transcripts. For biotinylated RNA generation, PCR products were used for in vitro transcription with the Biotin RNA Labeling Mix and T7 RNA polymerase. Nuclear proteins were extracted with the Nuclear and Cytoplasmic Protein Extraction Kit. After incubation, binding, and washing, beads were boiled in SDS buffer, and retrieved protein was detected by standard Western blotting.

CatRAPID and RPIseq, online protein-RNA binding predictors, were used to test the potential binding of MDM2 to lincRNA-p21.

**Chromatin Immunoprecipitation and ChIP-Seq Assays**

For immunoprecipitation, cells were lysed in cell lysis buffer, and the whole-cell extracts were incubated with protein A Sepharose beads combined with antibodies against p53 or with control IgG for 6 hours at 4°C. The chromatin immunoprecipitation (ChIP) assay kit was used for ChIP-quantitative PCR assays, according to the manufacturer’s instructions.

ChIP-Seq was performed from HA-VSMCs following the NimbleGen protocols for ChIP and amplification with minor modifications. The ChIP DNA was converted into Illumina sequencing libraries following the NEBNext ChIP-Seq Sample Prep Master Mix Set1 protocol. Multiplex adaptor and TrueSeq indexes were used. Reads were aligned by the use of Bowtie2, and regions with enriched signal compared with input were identified with model-based analysis of ChIP-Seq, using the default and suggested threshold of 1E-5. The peaks for selected loci were visualized using integrative genomics viewer (Broad Institute).

**Injury-Induced Mouse Model of Carotid Artery Neointimal Hyperplasia**

Local lentivirus-mediated gene transfer into injured carotid arteries was performed as described previously. Briefly, C57BL/6j mice underwent metal wire injury of the common carotid artery (n=5 for each group). After local injury and heparin injection, 20 μL recombinant lentivirus Si-lincRNA-p21 (1×10⁸ UT/mL) and control siRNA (1×10⁸ UT/mL) were instilled into the common carotid artery and allowed to dwell for 30 minutes. Uninjured arteries were used as sham control.

For cell proliferation and apoptosis, immunofluorescence and TUNEL assays, respectively, were performed. Sections were incubated with antibodies against Ki67. TUNEL assays were performed on paraffin sections with the ApopTag Plus In Situ Apoptosis Fluorescein Detection Kit according to the manufacturer’s procedure.

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**Figure 4.** LincRNA-p21 modulates p53 activity by augmenting the p53/p300 interaction. A, Coimmunoprecipitation (Co-IP) assays to detect the interaction of p53, p300, and mouse double minute 2 (MDM2). HA-VSMCs were transfected with Si-hlincRNA-p21 or control siRNA (Cntl-siRNA) in the presence or absence of p53 or with control IgG for 6 hours at 4°C. The chromatin immunoprecipitation (ChIP) assay kit was used for ChIP-quantitative PCR assays, according to the manufacturer’s instructions.

B through E, ChIP-Seq assays were carried out using p53 antibodies in HA-VSMCs after lincRNA-p21 knockdown. ChIP indicates chromatin immunoprecipitation. B, Venn diagram showing p53 ChIP-Seq peaks in control or lincRNA-p21 knockdown cells. C, Distribution of reads obtained by ChIP-Seq in lincRNA-p21 knockdown or control HA-VSMCs at 4 loci highly associated with the p53–apoptosis pathway. D, Distribution of reads in lincRNA-p21 knockdown or control HA-VSMCs at 5 additional loci associated with the p53 pathway. E, ChIP-quantitative polymerase chain reaction (qPCR) assays. Mdm2, Puma, Bax, and Noxa promoter/enhancer regions containing p53-binding sites were quantified by qPCR in control or lincRNA-p21 knockdown cells. F, ChIP-qPCR assays. Mdm2, Puma, Bax, and Noxa promoter/enhancer regions containing p53-binding sites were quantified by qPCR in control or lincRNA-p21 knockdown cells with or without p53 overexpression. Fold change of enrichment was determined relative to IgG controls. All values are the average of at least 3 biological replicates, and data shown are means±SD. *P<0.01 vs control; #P<0.05 vs p53/Cntl-siRNA.
Clinical Inclusion Criteria
The coronary artery disease group contained patients with >80% coronary artery stenosis, and the control group contained patients without clinically significant coronary artery occlusion. All procedures were conducted in compliance with protocols approved by the Third Military Medical University Ethics Committee, and written informed consent was received from all participants. Details of all probands are presented in Tables II and III in the online-only Data Supplement.

Statistical Analysis
All data are expressed as mean±SD unless otherwise stated. Data from each experiment were individually analyzed, and statistics were applied.
Figure 6. Continued
We used the Student unpaired t test to compare 2 independent groups. In experiments comparing multiple time points, separate t tests were used for each time point. For a comparison of ≥3 groups, 1-way ANOVA was used. GraphPad Prism 5.0 and SPSS 17.0 were used to perform the statistical analyses. Values of P<0.05 were considered significant.

An expanded Methods section is included in the online-only Data Supplement.

Results

LincRNA-p21 Regulates Cell Proliferation and Apoptosis

Given the vital role of p53 in the pathogenesis of atherosclerosis and the recent report that p53 regulates the expression of lincRNA-p21,30 we hypothesized that lincRNA-p21 is also involved in the development of atherosclerosis. We first examined the expression of lincRNA-p21 in aortic atherosclerotic plaques of ApoE−/− mice fed a high-fat diet, a widely used animal model of atherosclerosis. Indeed, we found that the expression of lincRNA-p21 was substantially lower in the aortic plaques of ApoE−/− mice compared with that of wild-type control mice, suggesting that lincRNA-p21 might play a role in atherosclerosis (Figure 1A).

Next, we investigated the function of lincRNA-p21 in cell proliferation and apoptosis. We used the mouse macrophage cell line RAW264.7 and the human VSMC line HA-VSMC, both of which have been widely used to study atherosclerosis in vitro. We designed small interfering RNA (siRNA) to inhibit mouse lincRNA-p21 and human lincRNA-p21 expression. The efficiency of siRNA transfection and the inhibition of endogenous lincRNA-p21 were tested and confirmed by quantitative RT-PCR (Figure 1B). Inhibition of lincRNA-p21 substantially increased total cell numbers in both RAW264.7 and HA-VSMC cells (Figure 1C). Increased cell proliferation and viability in these cells were further confirmed with an independent sensitive colorimetric assay (Figure 1D). Furthermore, lincRNA-p21 knockdown decreased apoptosis in both cell lines (Figure 1E and 1F). Together, these data indicate that lincRNA-p21 suppresses cell proliferation and induces apoptosis.

LincRNA-p21 Regulates the Expression of p53

Target Genes

To understand the molecular mechanism by which lincRNA-p21 regulates cell proliferation and apoptosis, we performed unbiased gene array analysis to measure gene expression changes in lincRNA-p21 knockdown cells. We found that 331 and 274 genes were upregulated and downregulated >2-fold, respectively, when lincRNA-p21 was knocked down (Figure 2A). Gene ontology analysis indicated that the downregulated genes were overrepresented for functional terms related to apoptosis, cell death, and the p53 signaling pathway (Figure 2A). Further examination revealed that many of the downregulated genes were previously reported p53 transcriptional targets (Figure 2B). We asked whether the expression of p53 itself was affected by lincRNA-p21 depletion. To our surprise, we detected no change in the expression of either p53 transcript or protein when lincRNA-p21 was inhibited (Figure 2C and 2D). We confirmed that si-lincRNA-p21 depletion in HA-VSMCs decreased the mRNA and protein levels of the p53 downstream target genes Puma,44,45 Bax,46 Noxa,47 and MDM2 (Figure 2E and 2F). Conversely, we overexpressed lincRNA-p21 in RAW264.7 cells (Figure 2G). Indeed, overexpression of lincRNA-p21 induced the expression of these p53 target genes, consistent with their role in the regulation of cell proliferation and apoptosis (Figure 2H and 2I).

LincRNA-p21 Interacts With MDM2

The above results indicated that lincRNA-p21 participates in the regulation of p53-dependent target gene expression without altering the expression level of p53 itself, suggesting that lincRNA-p21 might instead modulate the transcriptional activity of p53. Previous work showed that p300 and MDM2 play important roles in the regulation of p53 activity. P300 is an acetyltransferase that acetylates p53 and thereby enhances its activity. MDM2 antagonizes p53 by enhancing its degradation via the ubiquitin-proteasome pathway and by blocking its binding and acetylation by p300.35,36

The MDM2 protein contains several conserved structural domains, including an N-terminal p53 interaction domain and a C-terminal RING (really interesting new gene) domain (amino acid residues 430–480), which confers its E3 ubiquitin ligase activity. Intriguingly, the C-terminal RING domain also binds to RNA, indicating that MDM2 could function as an RNA-binding protein. To explore possible MDM2 binding to lincRNA-p21, we used computational approaches to assess the likelihood of protein-RNA interaction. catRAPID, a predictor of protein-RNA binding,37 predicted nucleotides 700 to 1500 of the lincRNA-p21 binding to the RING domain (amino acid residues 400–480) of MDM2 with the discriminative power of 82%. In contrast, the nt 1501 to 3000 region of the lincRNA-p21 presented no interaction signal (Figure 3A).

Next, we examined the direct binding of lincRNA-p21 and MDM2 through RIP in human HA-VSMC and mouse RAW264.7 cells. We used 2 independent anti-MDM2 antibodies to demonstrate the specificity of the interaction. RIP results showed that lincRNA-p21 interacts with MDM2 protein in both cell lines (Figure 3B and 3C). Deletion-mapping experiments showed that the nt 728-2057 region of lincRNA-p21 mediates the interaction with the RING domain of the MDM2 protein in mouse RAW264.7 cells (Figure 3D).

Figure 6. Continued LincRNA-p21 regulates p53-dependent cell proliferation and apoptosis in response to stresses. A and B, Relative cell number of HA-VSMCs at different time points was calculated after lincRNA-p21 knockdown without (A) or with (B) doxorubicin (Dox) treatment. Control (Cntl)-siRNA treatment serves as control. C, The mRNA levels of the indicated p53 target genes were determined by quantitative reverse transcription–polymerase chain reaction (qRT-PCR). D, The protein levels of total and acetylated p53 were determined by Western blotting. E, p53 chromatin immunoprecipitation (ChIP)–quantitative PCR (qPCR) assays. Mdm2, Puma, Bax, and Noxa promoter/enhancer regions were quantified by PCR in control or lincRNA-p21 knockdown cells with or without doxorubicin treatment. F, The protein levels of p300 were determined by Western blotting. G, P300 ChIP-qPCR assays. Mdm2, Puma, Bax, and Noxa promoter/enhancer regions were quantified by PCR in control or lincRNA-p21 knockdown cells with or without doxorubicin treatment. All values are the average of at least 3 biological replicates, and data shown are mean±SD. *P<0.01.
Figure 7. Inhibition of lincRNA-p21 results in increased neointima formation. A, Lentivirus vectors for lincRNA-p21 knockdown (si-miRNA-p21), or control siRNA (control) were in-site injected into the injured area of mouse carotid arteries. Sham operation serves as controls. Carotid arteries were harvested 30 days later after mice were fed a high-fat diet. Hematoxylin and eosin staining was performed to show the thickness of the neointima. B, Quantification of the intima-media thickness of sham-, control siRNA–, and si-lincRNA-p21–treated samples. C, Representative immunofluorescence images of Ki67 in mouse carotid arteries. DAPI staining marks cell nuclei. D, Quantification of the Ki67-positive signals of sham-, control siRNA–, and si-lincRNA-p21–treated samples. E, Representative immunofluorescence images of terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) staining in mouse carotid arteries. DAPI staining marks cell nuclei. F, Quantification of the TUNEL-positive signals of sham-, control siRNA–, and si-lincRNA-p21–treated samples. All values are the average of at least 3 biological replicates, and data shown are mean±SD. *P<0.05 vs control.
Figure 8. Role of lincRNA-p21 in p53-mediated atherosclerosis. A, Cell lysates from si-lincRNA-p21 (or control [Cntl]-siRNA)–treated carotid arteries were immunoprecipitated (IP) with p53 antibodies (Ab; or IgG to serve as a negative control), and associated proteins were detected with anti-p300 and MDM2 antibodies, respectively. Ten percent of the cell lysates were loaded to serve as controls. B, The mRNA levels of Mdm2, Puma, Bax, and Noxa were determined by quantitative reverse transcription–polymerase chain reaction (qRT-PCR) from si-lincRNA-p21 (or Cntl-siRNA)–treated carotid artery samples. C, RNAs isolated from coronary artery tissues of patients with coronary artery disease and aorta tissues of control patients were subjected to qRT-PCR assays to detect lincRNA-p21 expression levels. The solid and dashed horizontal lines represent standard deviation and mean, respectively. n=8 for each experimental groups. D, Schematic representation of a working model by which lincRNA-p21 feeds back on the function of p53 via binding to mouse double minute 2 (MDM2).
LincRNA-p21 Regulates the Transcriptional Activity of p53

To understand how the lincRNA-p21/MDM2 interaction affects the formation of the p53/p300/MDM2 complex, coimmunoprecipitation experiments were performed. We confirmed the interaction of p300 and p53 and of MDM2 and p53, consistent with prior reports. As expected, the interaction of p300 and p53 and of MDM2 and p53 increased in p53-overexpressing cells. LincRNA-p21 knockdown decreased p300/p53 interaction and increased MDM2/p53 interaction. In contrast, no interaction was detected when IgG was used for immunoprecipitation, demonstrating the specificity of the coimmunoprecipitation interaction assays (Figure 4A).

We next investigated whether inhibition of lincRNA-p21, which affected interactions among p300, MDM2, and p53 proteins without altering the p53 protein level, influenced p53 binding to the promoters/enhancers of its target genes. We performed p53 ChIP followed by high-throughput DNA sequencing (ChIP-Seq) experiments in HA-VSMCs treated with control or lincRNA-p21 siRNA. We obtained 36 to 53 million sequence reads from each experimental sample, and >95% of them uniquely aligned to the human genome (Figure I in the online-only Data Supplement). In control siRNA-treated samples, we identified >4800 p53-bound regions. Intriguingly, lincRNA-p21 knockdown diminished p53 binding at many of these regions (Figure 4B), suggesting that lincRNA-p21 is required for p53 to bind to many of its targets. We compared the peak distributions of Mdm2, Puma, Bax, and Noxa, 4 known p53-regulated genes, in control and lincRNA-p21 knockdown cells and found that knockdown of lincRNA-p21 dramatically reduced the association of p53 to the promoters/enhancers of these genes (Figure 4C). Similarly, we observed that lincRNA-P21 knockdown diminished the binding of p53 to promoters/enhancers of many additional targets (Figure 4D). ChIP-PCR assays further confirmed the above observation (Figure 4E). To verify that lincRNA-p21 functionally regulates p53 recruitment to target regulatory regions, we overexpressed p53 with or without knocking down endogenous lincRNA-p21 and measured the binding of p53 to the regulatory regions of target genes. ChIP results showed that p53 recruitment to its target promoters/enhancers was diminished when lincRNA-p21 was knocked down (Figure 4F). These observations suggest that lincRNA-p21, a transcriptional target of p53, can feed back and regulate the activity of p53, at least in part, by modulating the interaction of p53, p300, and MDM2.

LincRNA-p21 Modulates the Function of p53 in Regulating Cell Proliferation and Apoptosis

To investigate the interplay of p53 and lincRNA-p21 on cell proliferation, apoptosis, and atherosclerosis, we overexpressed p53 in human VSMC cells, with or without lincRNA-p21 knockdown. Whereas overexpression of p53 induced the expression of endogenous lincRNA-p21, lincRNA-p21 knockdown had no effect on p53 expression, consistent with the prior observations (Figure 5A). Overexpression of p53 inhibited VSMC proliferation and viability, evidenced by a dramatic decrease in direct cell counting (Figure 5B). Inhibition of lincRNA-p21 suppressed p53-mediated inhibition of VSMC proliferation, indicating that lincRNA-p21 modulates p53-dependent cell proliferation (Figure 5B). Similarly, p53-induced apoptosis was markedly repressed in lincRNA-p21 knockdown cells (Figure 5C). Moreover, lincRNA-p21 inhibited the stimulatory effect of p53 overexpression on levels of Mdm2, Puma, Bax, and Noxa (Figure 5D and 5E). Together, these results demonstrate that lincRNA-p21 functions through the p53 pathway to regulate cell proliferation and apoptosis.

It is well established that p53 regulates cell proliferation and apoptosis in response to stress. We tested whether the functional involvement of lincRNA-p21 and p53 in cell proliferation is regulated by treatment of doxorubicin, an anticancer chemotherapy drug that also causes cardiotoxicity. Knockdown of lincRNA-p21 increased whereas doxorubicin treatment reduced the number of cells in culture (Figure 6A and 6B), consistent with previous observation. Si-lincRNA-p21 partially suppressed the doxorubicin-induced reduction in cell number (Figure 6B). We further examined apoptosis and cell proliferation under such conditions. We found that doxorubicin treatment significantly induces apoptosis, marked by increased TUNEL staining, which is partially suppressed when lincRAN-p21 was knocked down (Figure IIA in the online-only Data Supplement). Conversely, doxorubicin treatment reduced cell proliferation, evidenced by decreased Ki67 labeling. Knockdown of lincRNA-p21 restored doxorubicin-inhibited cell proliferation (Figure IIB in the online-only Data Supplement). Next, we examined the expression of p53 target genes in cells treated with doxorubicin and Si-lincRNA-p21. We found that whereas doxorubicin treatment increased the expression of p53 target genes, knocking down endogenous lincRNA-p21 partially suppresses such an increase in gene expression (Figure 6C).

We asked whether knockdown of lincRNA-p21 might affect p53 protein level and its acetylation status under stress condition. We found that doxorubicin treatment induced the level of acetylated p53 significantly, which was reduced when endogenous lincRNA-p21 was inhibited (Figure 6D). Next, we investigated whether doxorubicin treatment and lincRNA-p21 knockdown could alter the binding of p53 to the promoters/enhancers of its target genes. ChIP-PCR showed that doxorubicin enhances the binding of p53 to its targets, which was reduced when lincRNA-p21 was knocked down (Figure 6E). In contrast, we found that doxorubicin treatment and lincRNA-p21 knockdown did not affect the expression level of p300 proteins (Figure 6F), nor did such treatment alter p300 binding to p53 target genes (Figure 6G). Together, these studies suggest that lincRNA-p21 modulates the activities and functions of p53 in regulating its target gene expression in response to stresses.

LincRNA-p21 Inhibits Neointima Formation in Carotid Arteries

Next, we investigated the involvement of lincRNA-p21 in the formation of neointima in vivo using the classic murine carotid artery injury model. Recombinant lentivirus
vector–expressing lincRNA-p21 siRNA or control siRNA was injected into the injured area of mouse carotid arteries. These mice were then fed a high-fat diet for 1 month, and neointima formation was examined. We verified the reduction in lincRNA-p21 expression in local-injury carotid tissues after lentivirus–si-lincRNA-p21 injection (Figure III in the online-only Data Supplement). Knockdown of lincRNA-p21 resulted in dramatic neointimal hyperplasia compared with controls (Figure 7A). Quantification of intima-media thickness confirmed a significant increase after si-lincRNA-p21 injection (Figure 7B). We asked whether inhibition of lincRNA-p21 affected cell proliferation and apoptosis in vivo. We performed immunostaining on vessel sections to detect the proliferation marker Ki67. The fraction of Ki67+ cells increased in siRNA–lincRNA-p21–treated vessels (Figure 7C and 7D). siRNA–lincRNA-p21–treated vessels also showed decreased apoptosis, as assessed with the TUNEL assay (Figure 7E and 7F).

To further uncover the role of lincRNA-p21 in the interaction of p53–p300–MDM2 proteins in vivo, we examined the effect of lincRNA-p21 knockdown on p300/p53 and MDM2/p53 interactions in carotid tissues. Consistent with in vitro studies, there was a reduced binding of p300 and p53 in si-lincRNA-p21–treated samples. Conversely, the association of MDM2 and p53 was increased in si-lincRNA-p21 samples (Figure 8A). As a result, the expression levels of the p53 targets Mdm2, Puma, Bax, and Noxa were repressed in si-lincRNA-p21–treated vessels in vivo (Figure 8B).

**Decreased LincRNA-p21 Expression in Patients With Coronary Heart Disease**

Finally, to determine whether dysregulated lincRNA-p21 expression is associated with coronary artery disease, we examined the expression of human lincRNA-p21 with quantitative RT-PCR assays using total RNAs isolated from coronary artery tissues of patients with coronary artery disease and artery tissues of control patients. Indeed, expression of lincRNA-p21 was >50% lower in patients with coronary artery disease compared with control patients (Figure 8C). Similarly, we tested the lincRNA-p21 level in another set of patients with coronary artery disease and control patients using total RNAs isolated from peripheral blood mononuclear cells, and we found that the lincRNA-p21 level was also decreased in patients with coronary artery disease (Figure IV in the online-only Data Supplement). These results implicate lincRNA-p21 in the development of atherosclerosis and coronary artery disease.

**Discussion**

In this study, we identified lincRNA-p21 as a key regulator of cell proliferation and apoptosis. We showed that lincRNA-p21 represses cell proliferation and induces apoptosis in vitro and in vivo. Knockdown of endogenous lincRNA-p21 accelerated neointima formation in injured carotid arteries. Mechanistically, we found that lincRNA-p21 directly binds to MDM2, leading to p53 release from MDM2 and binding to p300, which thus enhance p53 activity. This finding is significant because it implicates noncoding RNAs in cardiovascular diseases such as atherosclerosis and suggests that modulation of the activity of noncoding RNAs such as lincRNA-p21 may be a novel therapeutic approach to treat human cardiovascular disease.

It is well known that p53 plays an important role in the pathogenesis of atherosclerosis.1–6 The expression and transcriptional activity of p53 are tightly regulated at multiple levels. In particular, posttranscriptional regulation by ubiquitination and acetylation are known to be essential for the function of p53 proteins. MDM2, a direct transcriptional target gene of p53, appears to take part in both pathways. On one hand, p53 proteins can be degraded by MDM2 via the ubiquitination pathway.10,11 On the other hand, p53 is well known to be acetylated by the acetyltransferase p300, resulting in a dramatic induction of p53 activity.7–9 Interestingly, this effect is antagonized by p53 interaction with MDM2, which inhibits the formation of the p300–p53 complex. Clearly, this dynamic interaction among p300, MDM2, and p53 critically regulates the activity and function of p53 and positions MDM2 as a core regulator of p53 activity.21,51,52 A prior study showed that lincRNA-p21, which was identified as a transcriptional target of p53, feeds back to stimulate p53 function through its interaction with the RNA-binding protein hnRNP-K.33 Our study defines a second, MDM2-dependent mechanism by which lincRNA-p21 regulates p53 activity: LincRNA-p21 directly binds to MDM2, reducing p53–MDM2 interaction and increasing p53–p300 interaction. Consequently, the transcriptional activity of p53 is increased. Therefore, our investigation uncovered a novel mechanism by which p53 and lincRNA-p21 regulate each other’s expression and activity in a feedback manner (Figure 8D).

Of note, we observed that lincRNA-p21 uses its middle region (nt 728–2053) for the binding of MDM2. Huarte et al33 localized the hnRNP-K–binding region to the 5′ region (nt 1–778) of lincRNA-p21. Apparently, lincRNA-p21 uses 2 distinct regions to bind to 2 different proteins. It will be interesting to determine whether lincRNA-p21 binds to MDM2 and hnRNP-K simultaneously and whether binding to 1 protein modulates the binding to the other. Furthermore, it will be important to determine whether such an association is regulated in pathophysiological conditions, in particular the cardiovascular diseases. Are there functional correlations of lincRNA-p21 binding to both MDM2 and hnRNP-K?

The present study uncovered the involvement of lincRNA-p21 in atherosclerosis, at least in part, by interplaying with p53 in a feedback mechanism. It will be interesting to explore whether lincRNA-p21 can interact with epigenetic factors such as PRC1 and PRC2 to regulate the expression of atherosclerosis-related genes. Furthermore, we speculate that variants of lincRNA-p21 may be genetically linked to atherosclerosis. Given the existing of large numbers of lncRNAs and their important biological function, it is not unreasonable to predict that additional novel lncRNAs will be identified and linked to atherosclerosis and cardiovascular disease in the near future.

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Disclosures

None.

References

8. CW, Appella E. DNA damage activates p53 through a phosphorylation-acetylation of the p53 C-terminal domain.
Atherosclerosis is a major risk factor for cardiovascular disease. The cause of atherosclerosis is not fully understood. However, the proliferation of vascular smooth muscle cells and the formation of neointima are believed to contribute significantly to the development of atherosclerotic lesions. In this study, we identified a long noncoding RNA, lincRNA-p21, as a key regulator of atherosclerosis. We found that the expression of lincRNA-p21 was inhibited in atherosclerotic lesion in both mouse models and human patients. We show in our studies, using cultured cells and mouse models, that loss of lincRNA-p21 accelerates cell proliferation and the formation of atherosclerotic lesion. Conversely, overexpression of lincRNA-p21 inhibits cell proliferation and triggers apoptotic cell death. Thus, lincRNA-p21 could serve as a therapeutic target to treat atherosclerosis and related cardiovascular disorders.
LincRNA-p21 Regulates Neointima Formation, Vascular Smooth Muscle Cell Proliferation, Apoptosis, and Atherosclerosis by Enhancing p53 Activity

Gengze Wu, Jin Cai, Yu Han, Jinghai Chen, Zhan-Peng Huang, Caiyu Chen, Yue Cai, Hefei Huang, Yujia Yang, Yukai Liu, Zaicheng Xu, Duofen He, Xiaoqun Zhang, Xiaoyun Hu, Luca Pinello, Dan Zhong, Fengtian He, Guo-Cheng Yuan, Da-Zhi Wang and Chunyu Zeng

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Materials and Methods”

Online Supplementary Materials

Online Supplementary Materials and Methods

Cell culture, transfection, RNA interference, plasmids and DNA Damage Induction

Human vascular smooth muscle cell line HA-VSMC and mouse mononuclear macrophage cell line RAW264.7 were purchased from ATCC and cultured following the instructions of manufacturers. For RNAi, mouse lincRNA-p21 (mlincRNA-p21, AAAUAAAGAUUGUGAAUG), human lincRNA-p21 (hlincRNA-p21, CTGCAAGGCGCAGATGATGAdTdT), and negative control (NC) siRNAs (TTCTCCGAACGTGTCACGT) were synthesized by GenePharma biotechnology (Shanghai, China). siRNAs were transfected into cells at 100 nM final concentration using Lipofectamine 2000 and RNAiMAX (Invitrogen) according to manufacturer’s protocols. Full-length lincRNA-p21 was cloned into the pcDNA3.1(+) vector (Invitrogen) and transfected using Lipofectamine 2000. For DNA damage and endogenous p53 induction, cells were treated with doxorubicin hydrochloride (D1515, Sigma-Aldrich) at a final concentration of 1 µM for 18-24 hours.

RNA isolation and qRT-PCR analysis

Total RNAs were isolated using TRIzol reagent (Invitrogen) following manufacturer’s instructions. Reverse transcription (RT) was performed using M-MLV Reverse
Transcriptase (Invitrogen). Both random hexamers (Applied Biosystems) and oligo-dT (Invitrogen) were used for reverse transcription (RT) reactions. Real time quantitative PCR (qRCR) was performed using SYBR green master mix (Takara). Gapdh was used for normalization. PCR primer sequences are listed in Supplementary Table 1.

**Unbiased gene expression profiling**

Unbiased genome-wide transcriptome profiling was performed using the HUGENE 2.0 ST array (Affymetrix), which interrogates a total of 40,716 Refseq transcripts. Arrays were probed with cRNAs derived from HA-VSMCs treated with silincRNA-p21 or control siRNAs. Differentially expressed transcripts (greater than two fold differences) were identified and heatmap analysis was performed using Java Treeview. Gene functional annotation analysis was performed with the DAVID (Database for Annotation, Visualization and Integrated Discovery) tool.

**Antibodies**

For Western blotting, the following antibodies were used: mouse anti-p53 (sc-126, Santa Cruz), rabbit Acetyl-p53 (Lys382) (#2525, Cell Signaling), mouse MDM2 (sc-965, Santa Cruz), goat PUMA (sc-19187, Santa Cruz), rabbit Bax (sc-493, Santa Cruz), rabbit NOXA (sc-30209, Santa Cruz), rabbit cleaved caspase3 (#9661s, Cell Signaling). Mouse β-Actin (sc-47778, Santa Cruz) and mouse β-tubulin (T5168, Sigma-Aldrich) were used for normalization. For RNA immunoprecipitation (RIP) assays, antibodies 1 and 2 against MDM2 (MAB3776, Millipore, and sc-965X, Santa Cruz), respectively, were used. hnRNP-K antibody (04-088, Millipore) was used as a positive control. For Chromatin
Immunoprecipitation (ChIP) assays, ChIP Ab+ p53 antibody (17-613, Millipore), mouse p53 antibody (DO-1) (sc-126X, Santa Cruz), and rabbit p300 antibody (C-20) (sc-585X, Santa Cruz), were used. For Co-IP, p53 antibody (sc-126X, Santa Cruz) was used for immunoprecipitation (IP) and antibodies against P300 and MDM2 (sc-585 and sc-965, Santa Cruz) were used for immunoblotting (IB). For immunofluorescence, rabbit Ki67 antibody (ab15580, AbCam) was used. Mouse IgG antibodies (Millipore) were used as negative controls in RIP, ChIP, and Co-IP experiments.

**Cellular proliferation and apoptosis analysis**

Cells were seeded in 96-well plates, and cell proliferation was tested using the Cell Counting Kit-8 (CCK-8) assay Kit (C0038, Beyotime). Proliferating HA-VSMCs were quantified by Ki67 staining. For cellular apoptosis assay, HA-VSMCs were seeded in 48-well plates and TUNEL assay was performed using the ApopTag® Plus In Situ Apoptosis Fluorescein Detection Kit (Cat # S7111, Millipore).

**RNA immunoprecipitation, RIP assay**

RNA immunoprecipitation (RIP) experiments were performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (17-701, Millipore) in HA-VSMC and RAW264.7 cells following the manufacturer’s instructions. Two independent MDM2 antibodies were used. hnRNP-K antibody was introduced as a positive control. Briefly, 1-2×10^7 cells lysate was co-incubated with magnetic bead-antibody complex, and with rotating for 3 hours to overnight at 4°C. After washing 6-8 times, associated RNAs were isolated using phenol:chloroform:isoamyl alcohol (125:24:1, pH = 4.3) extraction.
Subsequently, co-precipitated RNAs were detected by qPCR and/or regular RT-PCR. Total RNAs from cell lysate (Input) were detected simultaneously as controls. Specific primer sequences used for detecting lincRNA-p21 are presented in Supplementary Table 1.

**Biotin RNA pull-down assay and deletion mapping**

RNA pull-down assay and deletion mapping were performed as described previously\(^{37}\). Briefly, the pcDNA3.1-lincRNA-p21 plasmid was used as template to synthesize biotinylated lincRNA-p21 transcripts. Specific PCR primer sequences were as follows:

- Forward PCR primers contained the T7 RNA polymerase promoter sequence (CCAAGCTTCTAATACGACTCACTATAGGGAGATGGCAGTCTGACCCACACTC),
- and Reverse primers for deleting lincRNA-p21 5’ ends 1-455nt: GAAAAAGCCTATCTCACC, 1-728nt: CTATGAAAGGGAGTCAGCAG, 1-2057nt: TGAGAAGTCACAGAAGCCAC, and full length: ACACGTGTGATGATTGTCT.

PCR products was purified using E.Z.N.A. Gel Extraction Kit and E.Z.N.A. cycle pure Kit (OMEGA). For biotinylated RNA generation, PCR products were used for *in vitro* transcription with the Biotin RNA Labeling Mix (Roche) and T7 RNA polymerase (Promega). In vitro transcribed RNA products were then treated with RNase-free DNase I (Life technology) and purified using the RNeasy Mini Kit (QIAGEN), the biotinylated RNA was heated to 60°C for 10min and slowly cooled to room temperature to facilitate secondary structure formation. Nuclear proteins were extracted from 4-5X10^7 mouse RAW264.7 cells using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) with the addition of 1 mM PMSF and protease inhibitors (Protease Inhibitor Cocktail Tablets, Roche). One milligram nuclear extracts were then mixed with 50 pmol of biotinylated
RNAs. Sixty microliters of washed streptavidin agarose beads (Invitrogen) were added in each reaction and incubated at room temperature for 1 hour. Beads were washed five times and boiled in SDS buffer, and retrieved protein was detected by standard western blotting.

**Immunoprecipitation and chromatin immunoprecipitation assays**

For immunoprecipitation (IP), cells were lysed in cell lysis buffer (Beyotime) and the whole cell extracts were incubated with protein A Sepharose beads (Invitrogen) combined with antibodies against p53, or with control IgG for 6 hours at 4°C. Beads were then washed and retrieved proteins were detected by immunoblotting using specific antibodies. The Chromatin Immunoprecipitation (ChIP) Assay Kit (17-295, Millipore) was used for ChIP-qPCR assays, according to the manufacturer’s instructions. Briefly, cultured cells were crosslinked with formaldehyde (10 min at 1%, 37°C). The chromatin contained cell lysates were sonicated into 200 to 1,000bp fragments and co-incubated with antibody-coated beads at 4°C overnight. The beads were washed 5 times with ChIP wash buffers. The retrieved DNAs were eluted, reverse-crosslinked, purified and analyzed by qPCR. Primer sequences were listed in Supplementary Table 1.

**ChIP-Seq**

ChIP-Seq was performed from HA-VSMC following NimbleGen protocols for chromatin immunoprecipitation and amplification with minor modifications. Briefly, 5-6x10⁷ cells were crosslinked with 1% formaldehyde for 10 min at RT. The reaction was stopped using 2.5M glycine at RT for 7 min. Nuclear lysates were sonicated to yield 100-1000 bp DNA
fragments (predominantly 200-500 bp) and co-incubated with p53-DO1 antibody at 4°C overnight. Protein G Dynabeads (10003D, Life Technologies) were blocked with PBS containing 1% BSA at 4°C. Blocked beads and nuclear lysates were then co-incubated for 4 hours at 4°C. The beads were washed 6 times with RIPA wash buffer. Precipitated DNAs were eluted, reverse-crosslinked, and purified. The ChIP DNA was then converted into Illumina sequencing libraries following the NEBNext ChIP-Seq Sample Prep Master Mix Set1 protocol (E6240, NEB). Multiplex adaptor and TrueSeq indexes were used. 50 nt single end sequencing was performed on an Illumina HiSeq 2000 (BGI, Shenzhen, China). Reads were aligned using Bowtie2 \(^{38}\), and regions with enriched signal compared to input were identified using MACS, using the default and suggested threshold of 1E-5 \(^{39}\). The peaks for selected loci were visualized using IGV (Broad Institute, MA).

**Injury-induced mouse model of carotid artery neointimal hyperplasia**

Local lentivirus-mediated gene transfer into injured carotid arteries was performed as described previously \(^{40, 41}\). Briefly, recombinant lentivirus vector pGLV-h1-GFP-puro expressing Si-RNA against lincRNA-p21 expression (Si-lincRNA-p21) and scramble control siRNA (Cntl-SiRNA) were constructed. C57BL/6J mice underwent metal wire injury of the common carotid artery (N=5 for each group). After local injury and heparin injection, 20ul of recombinant lentivirus Si-mlincRNA-p21 (1×10^8 UT/ml) and cntl-SiRNA(1×10^8 UT/ml) were instilled into the common carotid artery and allowed to dwell for 30 minutes. Uninjured arteries were used as sham control. Mice were killed after 30 days’ high fat diet induction and carotid arteries were collected, embedded, sectioned and stained with hematoxylin-eosin (H&E) to measure the degree of neointimal thickening.
The intima-media thickness ratios were calculated.

For cell proliferation and apoptosis, immunofluorescence and TUNEL assays, respectively, were performed. Sections were incubated with antibodies against Ki67 (1:200 dilution) followed by fluorescein conjugated secondary antibodies (1:400 dilution). Cell nuclei were stained with DAPI. Terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assays were performed on paraffin sections using the ApopTag® Plus In Situ Apoptosis Fluorescein Detection Kit (Cat # S7111 Millipore) according to the manufacturer's procedure. The use of mice for studies is in compliance with the regulations of the Third Military Medical University.

**Clinical inclusion criteria**

The coronary artery disease (CAD) group contained patients with greater than 80% coronary artery stenosis by coronary angiography. The control group contained patients without clinically significant coronary artery occlusion by coronary angiogram. Average ages were 58.25±10.90 years and 56.17±9.63 years for the CAD and control groups, respectively. Patients with diagnoses of hypertension or Type I/II diabetes were excluded. Coronary artery tissues from CAD patients and aorta tissues from control group were collected for RNA extraction (Supplemental Table 2). Additionally, we recruited a distincte cohort of coronary artery disease patients and controls (Supplemental Table 3). Total RNAs were isolated from peripheral blood mononuclear cells of these patients and control groups. All procedures were conducted in compliance protocols approved by the Third Military Medical University Ethics Committee, and written informed consent was received from all participants. Details of all probands are presented in Supplemental
Tables 2 and 3.

**Bioinformatics and statistical analysis**

CatRAPID\textsuperscript{42} and RPIseq\textsuperscript{43}, online protein-RNA binding predictors, were used to test the potential binding of MDM2 to lincRNA-p21.

All experiments were repeated at least three times. All data are expressed as mean ± SD unless otherwise stated. We used Student’s unpaired $t$-test to compare 2 independent groups. For a comparison of $\geq 3$ groups, 1-way ANOVA was used. GraphPad-Prism 5.0 (GraphPad-Software, San Diego, CA) and SPSS 17.0 (SPSS Inc, Chicago, IL) were used to perform the statistical analyses. Values of $p<0.05$ were considered significant.
Supplemental Figure 1. ChIP-Seq assays using p53 antibodies in HA-VSMC cells after lincRNA-p21 knockdown (or control siRNA to serve as controls). Total RNA sequencing reads (N) and the aligned rates to the human genome are listed.
Supplemental Figure 2. LincRNA-p21 regulates p53-dependent cell proliferation and apoptosis in response to stresses. HA-VSMC cells were transfected with Si-lincRNA-p21 (or cntl-SiRNA) and treated with doxorubicin (Dox) or without treatment in controls. (A) Representative immunofluorescence images of TUNEL staining and quantification of the TUNEL positive signals. (B) Representative immunofluorescence images of Ki67 staining and quantification of the Ki67 positive signals. DAPI staining marks cell nuclei.
Supplemental Figure 3. Knockdown endogenous mlincRNA-p21 in vivo in carotid arteries. Lentivirus vectors for lincRNA-p21 knockdown (Si-mlincRNA-p21), or control Si-RNA (control) were in-site injected into the injured area of mouse carotid arteries. Sham operation serves as controls. Carotid arteries were harvested 30 days later after feeding mice with high-fat diet and the expression of mlincRNA-p21 is determined by qPCR. Values are the average of 3 biological replicates and data shown are the mean±standard deviation (SD). * P<0.05 relative to control.
Supplemental Figure 4. Decreased lincRNA-p21 expression in patients with coronary artery disease. RNAs isolated from peripheral blood mononuclear cells of coronary artery disease patients and controls (normal) were subjected to qRT-PCR assays to detect lincRNA-p21 expression levels. n=8 for each experimental groups.
Supplemental Table 1 PCR primers used in this study

**Human qRT-PCR (5’-3’):**

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**Mouse qRT-PCR (5’-3’):**

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CCTGTCGTGATAGACTGTACCCCG

mBax (qPCR): GTTTTCATCCAGGATCGAGCAG
CCCCAGTTGAAGTTGCCATC

mNoxa (qPCR): TCGCAAAAAGAGCAGGATGAG
CCTTGTCTCAAATCTCCCG

mPuma (qPCR): GTACGAGCGCGCGAGACAAAG
GCACCTAGTTGGGCTCCATTTCTG

mGapdh (qPCR): GGGAAATTCAACGGCACAGT
AGATGGTGATGGGCTTCCC

P53-ChIP qPCR primers (5’-3’):

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Bax (ChIP): TAATCCCAACGCGCTTTGGAAAG
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Puma (ChIP): CTGTGGCCCTTTGTGCTGTAGTAC
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Noxa (ChIP): TTTTCTGGGCTTGTGTTACCC
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Supplemental Table 2: Control and Coronary Artery Patient Characteristics (used in Figure 8)

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