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LncRNA GAS5 regulates vascular smooth muscle cell cycle arrest and apoptosis via p53 pathway

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Abstract

Vascular remodeling is a pathological process following cardiovascular intervention. Vascular smooth muscle cells (VSMC) play a critical role in the vascular remodeling. Long noncoding RNAs (lncRNA) are a class of gene regulators functioning through various mechanisms in physiological and pathological conditions. By using cultured VSMC and rat carotid artery balloon injury model, we found that lncRNA growth arrest specific 5 (GAS5) serves as a negative regulator for VSMC survival in vascular remodeling. By manipulating GAS5 expression via adenoviral overexpression or short hairpin RNA knockdown, we found that GAS5 suppresses VSMC proliferation while promoting cell cycle arrest and inducing cell apoptosis. Mechanistically, GAS5 directly binds to p53 and p300, stabilizes p53-p300 interaction, and thus regulates VSMC cell survival via induction of p53-downstream target genes. Importantly, local delivery of GAS5 via adenoviral vector suppresses balloon injury-induced neointima formation along with an increased expression of p53 and apoptosis in neointimal SMCs. Our study demonstrated for the first time that GAS5 negatively impacts VSMC survival via activation the p53 pathway during vascular remodeling.

Keywords

LncRNA; GAS5; vascular remodeling; smooth muscle cell; p53

1. Introduction

Vascular remodeling is one of the major obstacles limiting the long-term clinical efficacy of cardiovascular intervention[1, 2]. Mechanical injury, such as angioplasty, bypass surgery, or transplantation arteriopathy, causes endothelial denudation, which triggers the production of inflammatory growth factors such as platelet-derived growth factor (PDGF), resulting in

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pathological proliferation of vascular smooth muscle cells (VSMCs) [3–5] and consequently neointima thickening and narrowing of the vessel lumen. Since VSMCs comprise the vast majority of neointimal mass, many efforts have been made to suppress VSMC proliferation in order to block neointimal hyperplasia after vascular injury [6–8]. However, although outside layer VSMC proliferation and inside layer VSMC apoptotic death are considered as an pair of balancing factors during neointima formation [9, 10], the molecular regulation of VSMC survival during vascular remodeling is relatively less focused.

Long non-coding RNAs (lncRNAs) are non-protein-coding RNA molecules longer than 200 nucleotides [11–13], which are encoded by 90% of the genome. Once the lncRNA gene is transcribed, spliced, and processed at the 5' and 3' ends, numerous lncRNAs are able to regulate target gene expression via different mechanisms ranged from chromatin modulation to protein degradation [14–16]. Recent studies have shown that lncRNAs have important functions in vascular development such as cardiomyocyte [17] and smooth muscle differentiation [18, 19] as well as cardiovascular diseases including cardiomyocyte hypertrophy [20], myocardial infarction [21], and atherosclerosis [22]. However, the function of lncRNAs in the mature VSMC in vascular remodeling is still poorly understood.

Growth Arrest Specific 5 (GAS5) is a well-known tumor suppressor lncRNA which is down-regulated in various cancers [23–25]. Although previous studies have reported the importance of GAS5 in cell proliferation and apoptosis, the detailed molecular mechanism is not well established. Published studies indicate that GAS5 regulates target gene expression through two mechanisms, i.e., 1) functioning as a microRNA sponge interacting with oncogenic miRNAs such as miR-21[26] and 2) serving as a protein sponge for growth-related transcription factors such as glucocorticoid receptor in regulating cell growth arrest [27]. Our recent studies show that GAS5 serves as a protein sponge in TGFβ signaling [28]. However, whether or not GAS5 regulates other molecular events in VSMC remain to be elucidated.

In this study, we demonstrated that GAS5 is a novel regulator for p53-mediated VSMC survival. GAS5 expression was down-regulated during VSMC proliferation and neointima formation. GAS5 suppressed VSMC proliferation, induced cell cycle arrest, and promoted VSMC apoptosis in primary cultured VSMC. Mechanistically, GAS5 bound p53 and p300, which stabilized p53 protein and thus promoted p53 function. Overexpression of GAS5 via adenoviral delivery suppressed neointima formation in rat carotid balloon injury model.

2. Materials and Methods:

2.1 Cells and Reagents:

Rat primary vascular smooth muscle cells (VSMCs) were cultured from rat thoracic aorta as described previously [29]. Rat thoracic aortas were removed and washed with PBS for 3 times. Briefly, aortic media layer was dissected, cut into pieces, and explanted onto a 6 well culture plate. Cells were growing at 37°C in a humidified atmosphere of 5% CO2 in DMEM supplemented with 10% FBS, penicillin and streptomycin for 2 weeks. VSMC phenotype were confirmed by expression of smooth muscle marker protein SM22α. PDGF-BB was obtained from R&D Systems (220-BB-010, Minneapolis, MN). Cycloheximide (01810) and

pifithrin-α (P4359) were obtained from Sigma-Aldrich (St. Louis, MO, USA). p53 (2524S), pCDK1 (4539), CDK1 (9112), HA-tag (2367), Flag-tag (14793S), p-p53 (9284) antibodies were purchased from Cell Signaling (Danvers, MA, USA). GAPDH (G8795) antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). PCNA (sc-56), Caspase 3 (sc-56053), NOXA (sc-56169), p21 (sc-6246), p300 (sc-48343), MDM2 (sc-965) antibodies was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

2.2 Animals and carotid balloon injury models:

All animals were housed under conventional conditions in the animal care facilities and received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals. All animal surgical procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia. Rat carotid artery balloon injury was performed using 2F Fogarty arterial embolectomy balloon catheter (Baxter Edwards Healthcare) as described previously [30]. 20–24 week old male Sprague Dawley rats (5 rats/group) were used in this study. Adenovirus expressing rat GAS5 shRNA was incubated at 5×10^9 pfu with balloon-injured carotid arteries for 20 min. 14 days later, the balloon-injured arteries were perfused with saline, fixed with 4% paraformaldehyde (PFA), embedded in paraffin, and sectioned. For quantification, neointima area were analyzed by Image J software. Intimal areas were calculated as the internal elastic lamina area minus luminal area. Average neointima area from 10 different fields were analyzed and shown as fold changes.

2.3 Construction of adenoviral vectors:

Adenoviral vector expressing GAS5 (AdGAS5) was constructed by cloning the rat GAS5 cDNA into the XhoI site of pShuttleIREShrGFP-1 (Agilent) and confirmed by sequencing. Adenoviral vector expressing rat GAS5 short hairpin RNA (shGAS5) was also constructed by using the following shRNA sequences: shGAS5 top strand: 5’-CGC GTC GGC CTT AGT CAC TAA CAA AGA GTT CAA GAG ACT CTT TGT TAG TGA CTA AGG CCT TTT TTC CAA A-3’; shGAS5 bottom strand: 5’-AGC TTT TGG AAA AAA GGC CTT AGT CAC TAA CAA AGA GTC TCT TGA ACT CTT TGT TAG TGA CTA AGG CCG A-3’. The viruses were purified as described previously [30]. Green fluorescent protein (GFP)-expressing adenovirus (Ad-GFP) was used as a control.

2.4 Quantitative RT-PCR (qPCR):

Total RNA was extracted from cells or tissues using Trizol reagent (Life Technologies, Gaithersburg, MD) and reverse transcribed to cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA). qPCR was performed using a Stratagene Mx3005 qPCR thermocycler (Agilent Technologies, La Jolla, CA). All reactions including no template controls were run in triplicate. After the reaction, the CT values were determined using fixed threshold settings. LncRNA expression was normalized to Cyclophilin (CYP). Primer sequences used in this study were listed in Supplementary Table S1.

2.5 RNA immunoprecipitation (RIP) assay:

RIP assay was performed as described [28]. Briefly, cells at 80–90% confluence in 15 cm^2 culture dishes were fixed with 1% PFA before scraped off, and then lysed in FA lysis buffer (50 mM HEPES, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100 (v/v), 0.1% sodium deoxycholate (w/v, pH7.5) containing 40 U/ml RNase inhibitor (Sigma-Aldrich, St. Louis, MO) and 1× Halt™ Protease Inhibitor Cocktail (Thermofisher Scientific, Grand Island, NY). After 4–6 rounds of sonication with 50% power output, 300 μl whole cell extract (around 500 μg total protein) was incubated with normal rabbit IgG or anti-p300 antibodies (1 μg) at 4°C overnight. The next day, the immunoprecipitates were captured with protein A/G agarose beads (50 μl) (Santa Cruz Biotechnology, Dallas, TX, USA). After washing with FA lysis buffer, samples were incubated with Proteinase K at 42°C for 1 hour to digest the proteins. The immunoprecipitated RNA was isolated, and the purified RNA was subjected to qRT-PCR analysis for detecting the presence of lncRNAs using respective primers.

2.6 Western blot:

Cultured cells or tissue samples were lysed in a RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% NP-40; and 0.1% SDS) by incubation at 4 °C with continuous rotation for 10 min. The cell lysates were centrifuged at 12 000×g for 10 min. The supernatant was collected, and the protein concentration was determined by BCA assay (Pierce, Rockford, USA). Protein extracts (10–50 μg) were dissolved on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS) plus 0.1% Tween-20 (TBST) at room temperature for 1 h followed by incubation with primary antibodies diluted in TBST at 4 °C overnight. After three 10-min washing with TBST, blots were incubated with the corresponding secondary antibody conjugated to HRP at room temperature for 1 h. The protein expression was detected with an enhanced chemiluminescent reagents.

2.7 Fluorescence in situ hybridization (FISH):

GAS5 RNA probe were synthesized and labeled using the FISH Tag RNA Multicolor kit (Life Technologies, Gaithersburg, MD). Carotid artery tissue cryosections or fixed primary cultured VSMC on cover glasses were digested with 20 μg/mL proteinase K at 37 °C for 1 hour and washed with 2× SSC solution and then water at room temperature each for 5 min. The sections were dehydrated, air-dried, and then incubated with pre-denatured GAS5 probes in a dark and humid environment at 55°C for 24 hours to allow hybridization. Slides were then washed in 50% formamide in 2× SSC for 4 times before mounting. Nuclei were counterstained with 5,6-diamidino-2-phenylindole (DAPI).

2.8 MTT cell proliferation assay:

Cell proliferation was evaluated with 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) assay using a TACS MTT Cell Proliferation Assay Kit (Trivegen). The optical density at 570 nm was measured.

2.9 Cell cycle analysis:

1×10^6 cells were harvested and resuspended in 500 μl of reaction buffer containing 1 μl of Nuclear-IDTM Red dye (Nuclear-IDTM Red Cell Cycle Analysis Kit, Enzo Life Sciences, USA). After mixing, cells were incubated for 15 min in the dark. Cell cycle analysis was performed on a FACSCalibur™ (Becton Dickinson) and analyzed by the Flowjo™ software.

2.10 Cell apoptosis analysis:

In vivo cell apoptosis was evaluated by detecting DNA fragmentation using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP–digoxigenin nick end-labeling method (TUNEL kit, Roche, USA). In vitro cell apoptosis was measured by Flow Cytometry. Cells were stained with both Annexin V-Pecific Blue (BD Biosciences) and propidium iodide (PI) and analyzed on a FACSCalibur™ (Becton Dickinson). The percentages of positive-stained cells were quantified using Flowjo™ software (Becton Dickinson).

2.11 Immunohistochemistry (IHC) staining:

For IHC staining, tissue sections were rehydrated, permeabilized with 0.01% Triton X-100 in PBS, blocked with 10% goat serum, and incubated with primary antibodies at 4°C overnight followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody. The sections were counterstained with hematoxylin.

2.12 Phylogeny analysis:

Human GAS5 DNA sequences were retrieved from NONCODE database. Mouse and rat GAS5 DNA sequences were retrieved from NCBI. Alignment and phylogeny tree construction was built by using Geneious V11. For the alignment, Geneious global alignment with a default setting was used. For the phylogeny tree construction, tamura-nei model and neighbor-joining method were used to calculate distance. Populus trichocarpa 4CL gene served as the outgroup.

2.13 Statistical analysis:

Each experiment was repeated for at least three times. All values are presented as means ± SEM. Comparisons of parameters between two groups were made by unpaired Student's t-tests. P values from 0.01 to 0.05 or lower than 0.01 were considered significant (*) or very significant (**), respectively.

3. Results

3.1 GAS5 suppressed VSMC proliferation.

GAS5 is a well-established tumor suppressor whose expression is down-regulated in various cancers [23, 31]. Since neointimal hyperplasia is caused by abnormal VSMC proliferation, we first assessed GAS5 expression in proliferative VSMC. Primary VSMCs were cultured in DMEM medium containing 10% FBS (Control), treated with 10 ng/ml of PDGF-BB or serum starvation for 24 hours. Cell proliferation was assessed by the expression of proliferating cell marker PCNA (Fig 1A–1B). GAS5 expression was inversely correlated with PCNA expression (Fig 1C). Decreased GAS5 expression in PDGF-BB-treated VSMC

and increased GAS5 expression in serum-starved VSMC were observed via RNA fluorescence *in situ* hybridization (RNA-FISH) (Fig 1D). These results indicated that GAS5 was inversely correlated with VSMC proliferation.

To determine if GAS5 regulates VSMC proliferation, we overexpressed and knocked down GAS5 by transducing AdGAS5 or AdshGAS5 into VSMCs, respectively. VSMCs transduced with control (AdGFP), AdGAS5, or AdshGAS5 were cultured in DMEM medium containing 10% fetal bovine serum (FBS) for two days followed by tetrazolium-based colorimetric assay (MTT assay) to measure cell proliferation rate. As shown in Fig 1E, overexpression of GAS5 suppressed VSMC proliferation while GAS5 knockdown increased the cell proliferation. Consistently, PCNA expression was suppressed by AdGAS5 but enhanced by AdshGAS5 (Fig 1F–1I). These results demonstrated that GAS5 suppresses VSMC proliferation in vitro.

3.2 GAS5 induced VSMC cell cycle arrest.

VSMC proliferation and apoptotic death are considered as a pair of balancing factors regulating VSMC mass during neointima formation [32, 33]. Cell growth relies on cell cycle progression. To assess the role of GAS5 in cell cycle regulation, we overexpressed or knocked down GAS5 by transducing adenoviral vectors expressing GAS5 cDNA or shRNA into VSMCs. The flow cytometry analysis using nuclear-IDTM Red dye (Nuclear-IDTM Red Cell Cycle Analysis Kit, Enzo Life Sciences, USA) staining showed that overexpression of GAS5 arrested VSMC in G0/G1 phase, accompanying with an increased population in sub-G1 phase. Conversely, knockdown of GAS5 promoted cell cycle progression, resulting in an increased VSMC population in G2/M-phase (Fig 2A). The function of GAS5 in cell cycle arrest was also revealed by CDK1 phosphorylation, a key G1-S transition regulator in cell cycle. As shown in Fig 2B–2E, overexpression GAS5 suppressed CDK1 phosphorylation while knockdown of GAS5 increased CDK1 phosphorylation, even in the serum-starvation condition. These results demonstrated that GAS5 arrests VSMC cell cycle at G0/G1 stage.

3.3 GAS5 induced VSMC apoptosis.

Cell cycle analysis suggested that GAS5 also induced VSMC cell death (Fig 2A). To confirm if GAS5 is involved in VSMC apoptosis, we performed Annexin V-Pacific blue/PI double staining. As shown in Fig 2F, overexpression of GAS5 increased the overall apoptotic cell population including the early (AnnexinV+PI-) and late apoptotic cells (Annexin-V+PI+). This effect of GAS5 was further revealed by the increased cleavage of Caspase 3. As shown in Fig 2G–2H, overexpression of GAS5 in VSMC significantly enhanced the Caspase 3 cleavage. In contrast, knockdown of GAS5 suppressed Caspase 3 cleavage (Fig 2I–2J). These results demonstrated that GAS5 promotes both cell cycle arrest and apoptosis in VSMC.

3.4 GAS5 induced cell cycle arrest and apoptosis via activation of p53 pathway.

Since p53 is a master regulator for both cell cycle arrest and cell apoptosis [34], we examined if GAS5 regulates p53 signaling to affect VSMC survival. Overexpression of GAS5 increased p53 level while knockdown of GAS5 suppressed p53 expression in VSMC

(Fig 3A–3D). To assess the effect of GAS5 on p53 signaling pathway, p53 downstream genes BAX, PUMA, NOXA and p21 mRNA expression were analyzed by RT-qPCR in VSMCs transduced with AdGFP or AdGAS5. As shown in Fig 3E, overexpression of GAS5 increased the expression of all the p53 downstream genes detected. Since NOXA is an upstream regulator for Caspase 3 cleavage involved in cell apoptosis, and p21 is a canonical cyclin-dependent kinase inhibitor for CDK1 phosphorylation and cell cycle regulation, we further detected NOXA and p21 protein expression. As shown in Fig 3F–3G, overexpression of GAS5 increased while knockdown of GAS5 suppressed NOXA and p21 protein expression (Fig 3H–3I).

In order to further determine if GAS5 regulated NOXA and p21 expression through p53 pathway, we blocked p53 activity using its inhibitor Pifithrin α (PFT-α). As shown in Fig 3J–3K, PFT-α treatment dramatically suppressed GAS5-induced NOXA and p21 expression. Consistently, PFT-α treatment impeded GAS5 activity in suppressing VSMC proliferation (Fig 3L) and rescued VSMC from GAS5-induced apoptosis (Fig 3M). Collectively, these results indicated that GAS5 suppressed VSMC growth while induced cell apoptosis through p53 pathway.

3.5 GAS5 suppressed p53 ubiquitination and regulated p53 turnover.

Since p53 level is mainly regulated via post-translational mechanisms[35], we first detected if GAS5 regulated p53 transcription. Thus, p53 mRNA expression in VSMCs transduced with AdGAS5 for 0, 0.5, 1, 6 and 12 hours was detected. Interestingly, overexpression of GAS5 did not alter p53 mRNA level (Fig 4A), suggesting that GAS5 may regulate p53 at post-transcriptional level. To test if GAS5 affects p53 protein turnover, we used protein translation inhibitor cycloheximide (CHX) to block *de novo* protein synthesis in AdGFP or AdGAS5-transduced VSMCs. As shown in Fig 4B–4C, CHX treatment caused p53 reduction due to the internal protein degradation. However, p53 level maintained at a relatively stable level from 0 to 12 hours in AdGAS5-transduced VSMC, suggesting that GAS5 promoted p53 stability. Phosphorylation is a common mechanism to stabilize p53 protein [36]. Therefore, we tested if GAS5 promotes p53 phosphorylation. As shown in Fig 4D–4E, overexpression of GAS5 induced p53 phosphorylation in VSMCs.

It is reported that p53 turnover is regulated by the ubiquitination followed by protein degradation [37]. Therefore, we determined if GAS5 is involved in p53 ubiquitination by performing an *in vitro* ubiquitination assay. Plasmid carrying HA-tagged ubiquitin and Flag-tagged p53 were co-transfected into AdGFP or AdGAS5-transduced VSMCs. 48 hours after the transfection, co-immunoprecipitation (Co-IP) was performed using anti-Flag antibody to pull down p53. Ubiquitinylated-p53 was then detected using anti-HA antibody. As shown in Fig 4F, overexpression of GAS5 decreased p53 ubiquitinylation level.

To determine the molecular mechanism by which GAS5 regulates p53 stability, we predicted the potential interactions of GAS5 with p53 and p300 through catRAPID fragments (http://s.tartaglialab.com/update_submission/158824/beeef3b8ee, sFig 1). To confirm the interactions among GAS5, p53 and p300, an RNA immunoprecipitation (RIP) assay was performed. VSMCs were cultured in DMEM with 10% FBS or in serum-deprived condition for 24 hrs. RNA-protein complexes from VSMCs were pulled down using p53 or p300

antibody. GAS5 was then detected by quantitative RT-PCR. As shown in Fig 4G–4H, GAS5 indeed bound p53 and p300. Serum starvation enhanced the GAS5 binding to p53 and p300. These data suggested that GAS5 stabilizes p53 in VSMCs via recruiting p300. To confirm the role of GAS5 in promoting p53-p300 interaction, Co-IP was performed in VSMCs transduced with Ad-GFP or Ad-GAS5. As shown in Fig 4I, overexpression of GAS5 increased p53-p300 interaction. These results demonstrated that GAS5 suppresses p53 ubiquitination and inhibiting p53 turnover through recruiting p300.

MDM2 is a key E3 ligase for p53 ubiquitination and degradation [36, 38]. We thus tested if GAS5 regulated p53 stability through MDM2 pathway. Surprisingly, overexpression of GAS5 in VSMC, which increased p53 level, increased MDM2 mRNA (sFig 2A) and protein expression (sFig 2B–2C), suggesting that MDM2 failed to cause p53 degradation in GAS5-overexpressed VSMCs. Moreover, MDM2 inhibitor Mi733 (10 μM) or AMG232 (10 μM) did not increase p53 protein level (sFig 2D–2E). These data indicated that GAS5 promoted p53 stability in a MDM2-independent manner.

3.6 GAS5 suppressed neointima formation in injured rat carotid artery.

Our *in vitro* experiments demonstrated that GAS5 promoted VSMC growth arrest and apoptosis via increasing p53 stability and enhancing its activity. Since VSMC proliferation and survival are the major events for neointima formation [39], we further explored the role of GAS5 in neointima VSMC survival *in vivo* by using the rat carotid artery balloon injury model. GAS5 expression was significantly attenuated in injured carotid artery VSMCs (Fig 5A). Consistently, GAS5 messenger level was also dramatically decreased in carotid artery following the injury (Fig 5B). To determine if GAS5 is important for VSMC proliferation and the neointima formation *in vivo*, we used adenoviral vector (AdGFP or AdGAS5) to deliver GAS5 to the injured arteries. 14 days following the injury, animals were euthanized, and the carotid arteries were fixed and embedded for histological analyses. As shown in Fig 5C–5D, overexpression of GAS5 suppressed neointima formation. VSMC proliferation was also inhibited as shown by the diminished PCNA-positive cells in GAS5-expressed arteries (Fig 5E–5F). Meanwhile, we also detected increased p53 expression in neointima VSMCs due to GAS5 overexpression (Fig 5G–5H). Since GAS5 induced VSMC apoptosis through p53 pathway *in vitro*, we also performed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to detect cell apoptosis *in vivo*. As shown in Fig 5I–5J, overexpression of GAS5 dramatically increased the number of apoptotic VSMC in the neointima. These results confirmed that GAS5 suppressed neointima formation via inhibition of VSMC proliferation and induction of VSMC apoptosis.

4. Discussion

In this study, we provided evidence for the first time that lncRNA GAS5 functions as a regulator for p53 pathway in VSMC survival during neointima formation. GAS5 was down-regulated in proliferating VSMCs. GAS5 suppressed cell proliferation while promoted cell apoptosis and cell cycle arrest. Mechanistically, GAS5 bound p53 and its activator p300, which enhanced p53 protein stability and its activity in VSMCs, causing increased expression of cell cycle inhibitors and apoptosis activating genes and consequently leading

to VSMC cell cycle arrest and increased apoptosis (Fig 6). Importantly, overexpression of GAS5 via adenoviral delivery suppressed neointima formation in rat carotid balloon injury model, which correlated with the increased p53 expression and enhanced apoptosis in neointimal VSMCs.

As the master regulator in multiple cellular progresses, p53 and its downstream signaling have been reported to interact with the lncRNA regulatory network. On the one hand, p53 regulates the expression of lncRNAs, such as lncRNA-p21, PANDA, and H19, at the transcription level [40–42]. On the other hand, p53 signaling is regulated by a few lncRNAs, such as MALAT1 and MEG3, during cell proliferation and apoptosis [43, 44]; suggesting that p53 is an important node in lncRNA-related gene regulatory network. As one of the most well-established tumor suppressive lncRNA, GAS5 is involved in p53 signaling under different tumor-related circumstances. In nonsmall cell lung cancer, GAS5 regulates cell growth arrest and induces apoptosis through p53- or E2F1-dependent pathways [45]. While in stomach cancer, GAS5 directly regulates p53 downstream gene p21 expression [46]. Our study provided the first evidence that GAS5 recruits p300 to protect p53 protein from degradation and thus promotes VSMC cell growth arrest and apoptosis in vascular remodeling. p300 is one of the most well-established p53 regulators mediating p53 acetylation and stabilizing p53 [47].

Interestingly, a substantial level of GAS5 was observed in quiescent VSMC (Fig 1D & Fig 5A), which rise a question about the function of GAS5 in normal cells. One possibility is that in normal VSMCs, GAS5 regulates p53 signaling to maintain chromosome stability, which is supported by the fact that p53 is also expressed in quiescent VSMC (Fig 4B). An alternative hypothesis is that in quiescent VSMCs, GAS5 is occupied by other protein co-factors such as the glucocorticoid receptor. But in the pro-apoptotic response, the increased p53 and p300 competitively bind GAS5, resulting in activation of p53 signaling (Fig 4G–4H). Future studies could identify other GAS5-associated proteins and their functions in physiological and pathological conditions.

Our previous studies show that mouse GAS5 in SMC progenitor cells binds to Smad3 as a protein sponge and suppresses TGF-β-induced VSMC differentiation [28]. Herein, we identified a novel function of GAS5 for mature VSMC survival. Since VSMC is not terminally differentiated but retains remarkable phenotypic plasticity [48–50] and can undergo phenotypic alteration from contractile VSMC to synthetic VSMC during injury-induced vascular remodeling [5, 51–53], whether or not GAS5 is involved in mature VSMC phenotypic modulation or re-differentiation require further investigation. Interestingly, a previous publication indicates that p53 may function in VSMC osteogenic phenotypic switch, suggesting that GAS5 could regulate mature VSMC phenotype via p53 signaling pathway [54]. However, detailed functional analyses are required to test the potential role of GAS5-p300-p53 axis in mature VSMC phenotype in the future.

Interestingly, human GAS5 has nearly 30 splice variants while rat GAS5 only has two annotated transcripts sharing the identical primary sequence. By comparing the rat GAS5 with human variants (sFig 3), we found that human variant 5 (hGAS5v5) and variant 16 (hGAS5v16) contain the most similar primary sequence as rat GAS5. Both hGAS5v5 and

hGAS5v16 were highly expressed in VSMC (sFig 4A) and directly bound p53 and p300 (sFig 5). However, human GAS5 orthologs were not regulated by PDGF-BB (sFig 4B–4C) while decreased by serum starvation (sFig 4D–4E). Serum starvation also inhibited GAS5 dissociation with p53 and p300 (sFig 5). These effects are opposite to rat GAS5, suggesting that the function or biochemical properties of GAS5 may be species- and cell context-depend.

Nevertheless, we identified GAS5 as a novel regulator for promoting p53-mediated VSMC cell cycle arrest and apoptosis, which may render GAS5 as a potential agent to develop RNA-based treatment for injury-caused vascular remodeling.

Resource	Source	Identifier
Reagents		
Adenoviral vector expressing rat GAS5	**PI's lab**	**N/A**

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- LncRNA GAS5 is a negative regulator for vascular smooth muscle cell (SMC) survival.
- GAS5 suppresses SMC proliferation while promoting cell cycle arrest and inducing cell apoptosis.
- GAS5 regulates SMC survival by stabilizing p53-p300 interaction to induce p53-downstrem genes.
- GAS5 promotes VSMC apoptosis via activation the p53 pathway during vascular remodeling.

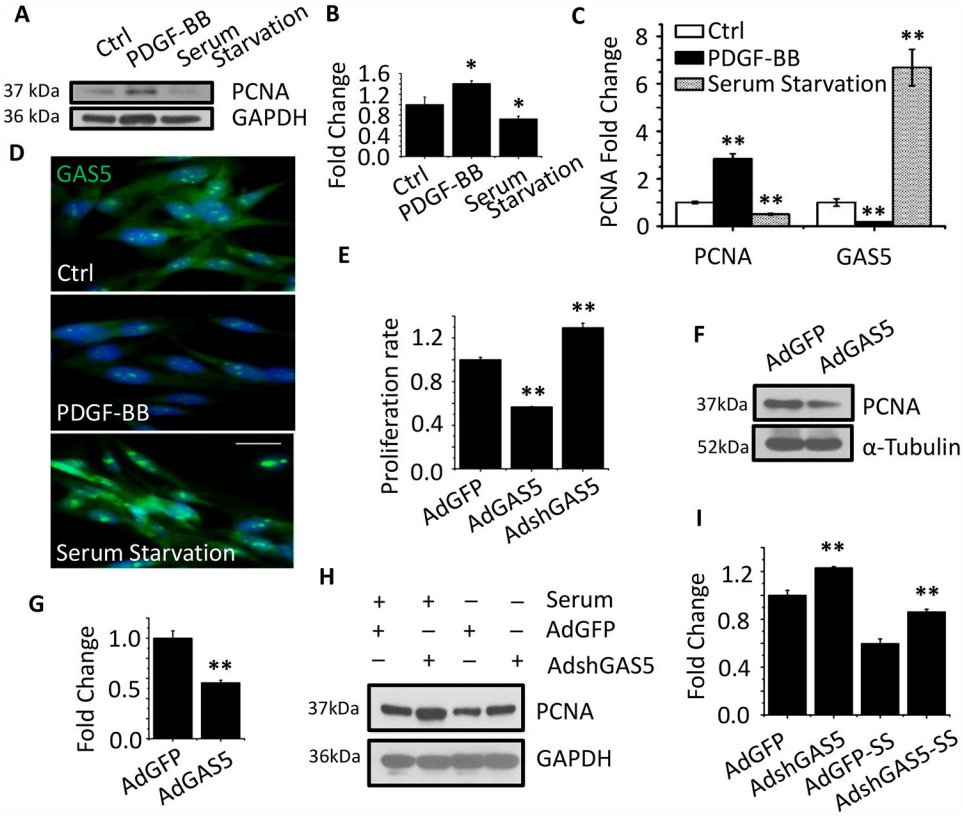

Figure 1: LncRNA GAS5 suppressed VSMC proliferation.

A) PDGF-BB (10 ng/ml) stimulated while serum-starvation inhibited PCNA expression. Cells were treated for 24 h. Ctrl: VSMC cultured in DMEM medium with 10% FBS. **B)** Quantification of PCNA protein levels in A by normalizing to GAPDH. **C)** GAS5 was inversely correlated with PCNA expression. VSMCs were treated with PDGF-BB (10 ng/ml) or serum-starvation for 24 h. Relative RNA expression was normalized to cyclophilin. **D)** GAS5 expression in PDGF-BB-treated and serum-starved VSMCs, as shown by RNA-FISH. VSMCs were treated with PDGF-BB (10 ng/ml) in 10% FBS DMEM or serum-starved for 12 h. Bar: 20 μm. **E)** GAS5 suppressed VSMC proliferation. VSMCs were transduced with adenoviral vector expressing GAS5 (AdGAS5) or its shRNA (AdshGAS5) for 2 days, relative VSMC proliferation rates were assessed by MTT assay and normalized to control vector (AdGFP)-transduced cells. **F)** Overexpression of GAS5 suppressed PCNA protein expression. **G)** Quantification of PCNA expression in F by normalizing to α-Tubulin. **H)** Knockdown of GAS5 enhanced PCNA expression. VSMCs were transduced with AdGFP or AdshGAS5 for 2 days and then cultured with or without 10% FBS for 24 hours. **I)** Quantification of PCNA expression in H by normalizing to GAPDH. SS: serum starvation. * $p<0.05$; ** $p<0.01$; n=3 for all panels.

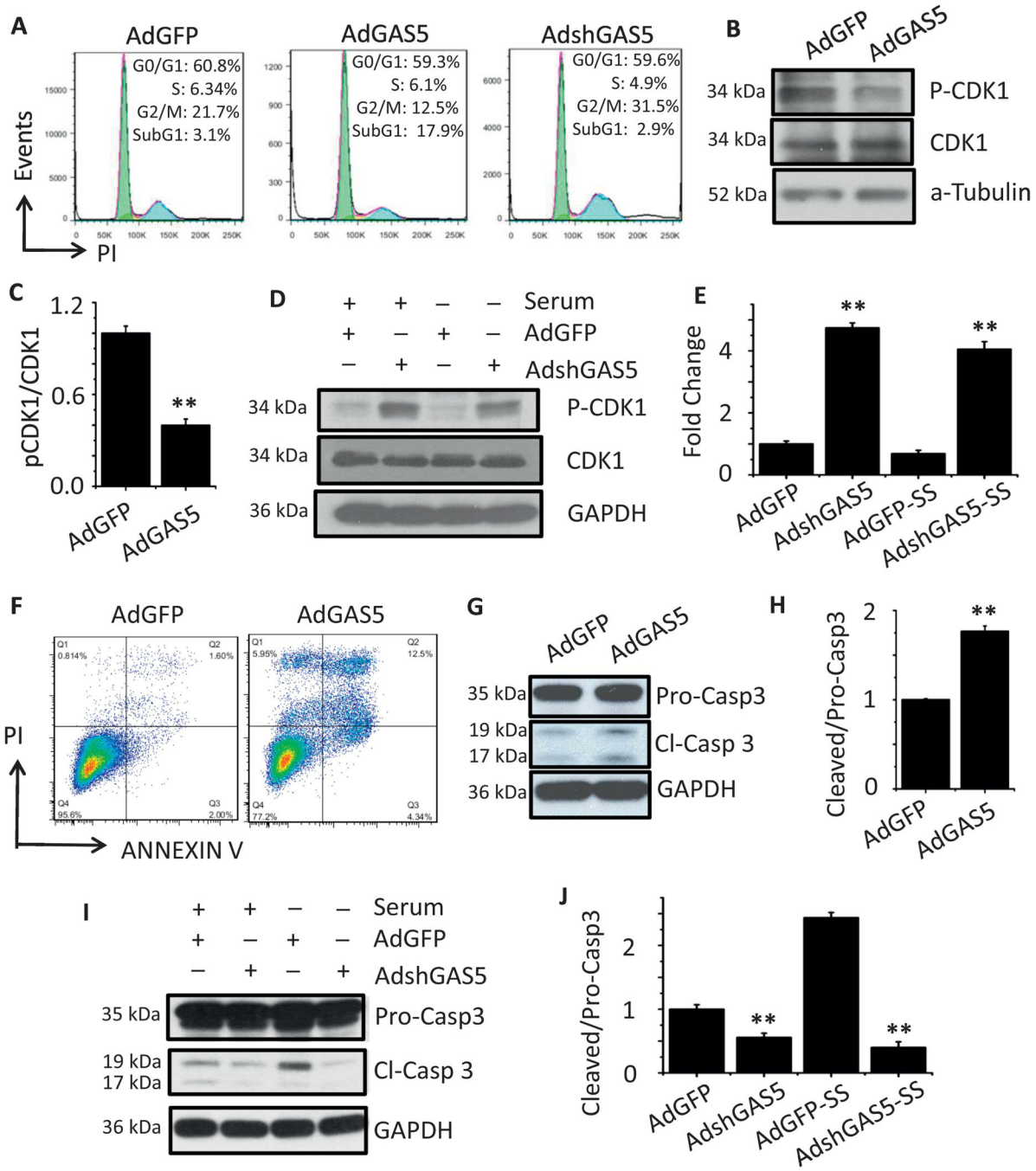

Figure 2: GAS5 induced VSMC cell cycle arrest and apoptosis.

VSMCs were transduced with AdGFP, AdGAS5, or AdshGAS5 as indicated for 2 days. **A)** GAS5 induced VSMC cell cycle arrest. Cell cycles were assessed by flow cytometry with PI staining (FACS). **B-C)** GAS5 suppressed CDK1 phosphorylation. CDK1 phosphorylation was assessed by Western blot (B) and quantified (C) by normalizing to total CDK1 level. **D)** Knockdown of GAS5 increased CDK1 phosphorylation. VSMCs were cultured with or without 10% FBS for 24 h following the adenovirus transduction as indicated. **E)** Quantification of CDK1 phosphorylation in D by normalizing to total CDK1. **F)** GAS5

induced VSMC apoptosis, as assessed by FACS after Annexin V/PI staining. **G-H)** Overexpression of GAS5 induced Caspase 3 cleavage. Cleaved caspase 3 (Cl-Casp 3) was assessed by Western blot (G) and quantified by normalizing to pro-Caspase3 (H). **I)** Knockdown of GAS5 suppressed Caspase 3 cleavage. VSMCs were cultured with or without 10% FBS for 24 hours following the adenovirus transduction as indicated. **J)** Quantification of Caspase3 cleavage in I by normalizing to pro-Caspase 3. * $p<0.05$; ** $p<0.01$; n=3.

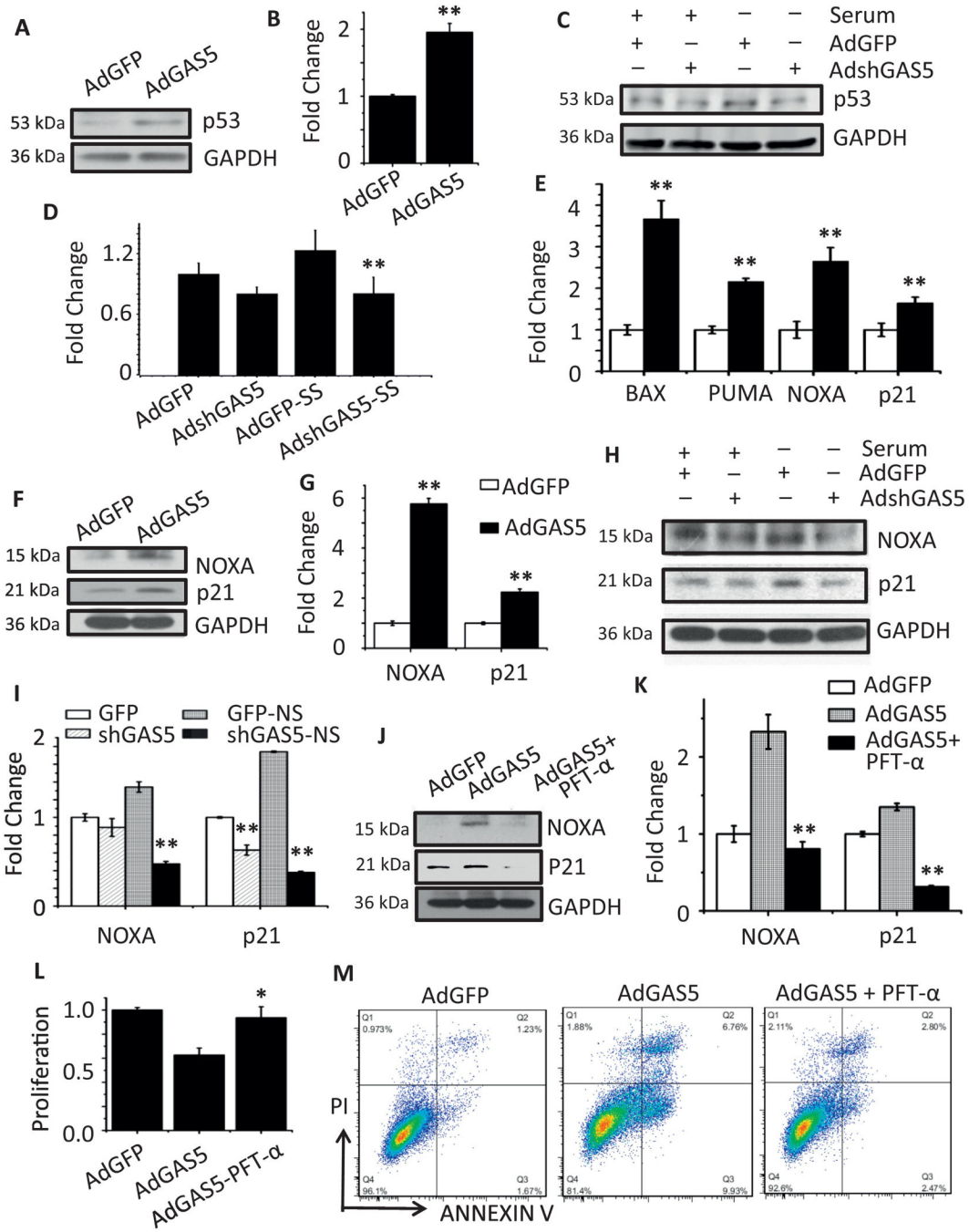

Figure 3: GAS5 induced cell cycle arrest and apoptosis via activation of p53 pathway.

A) Overexpression of GAS5 increased p53 expression in VSMCs. **B)** Quantification of p53 level in A by normalizing to GAPDH. **C)** Knockdown of GAS5 by its shRNA decreased p53 expression in VSMCs. VSMC were serum-starved for 48 h to induce p53 expression. **D)** Quantification of p53 level in C by normalizing to GAPDH. **E)** Overexpression of GAS5 increased the mRNA expression of p53 down-stream genes. **F)** Overexpression of GAS5 increased Noxa and p21 protein expression. **G)** Quantification of protein expression in F by normalizing to GAPDH. **H)** Knockdown of GAS5 decreased Noxa and p21 protein

expression. **I)** Quantification of protein expression in H by normalizing to GAPDH. **J)** p53 inhibitor pifithrin (PFT-α, 10 μM for 48 h) suppressed GAS5-induced Noxa and p21 expression. **K)** Quantification of protein expression in J by normalizing to GAPDH. **L)** PFT-α (10 μM for 48 h) reversed GAS5-mediated VSMC growth arrest as assessed by MTT assay. **M)** PFT-α (10 μM for 48 h) suppressed GAS5-induced VSMC apoptosis. * $p<0.05$; ** $p<0.01$; n=3.

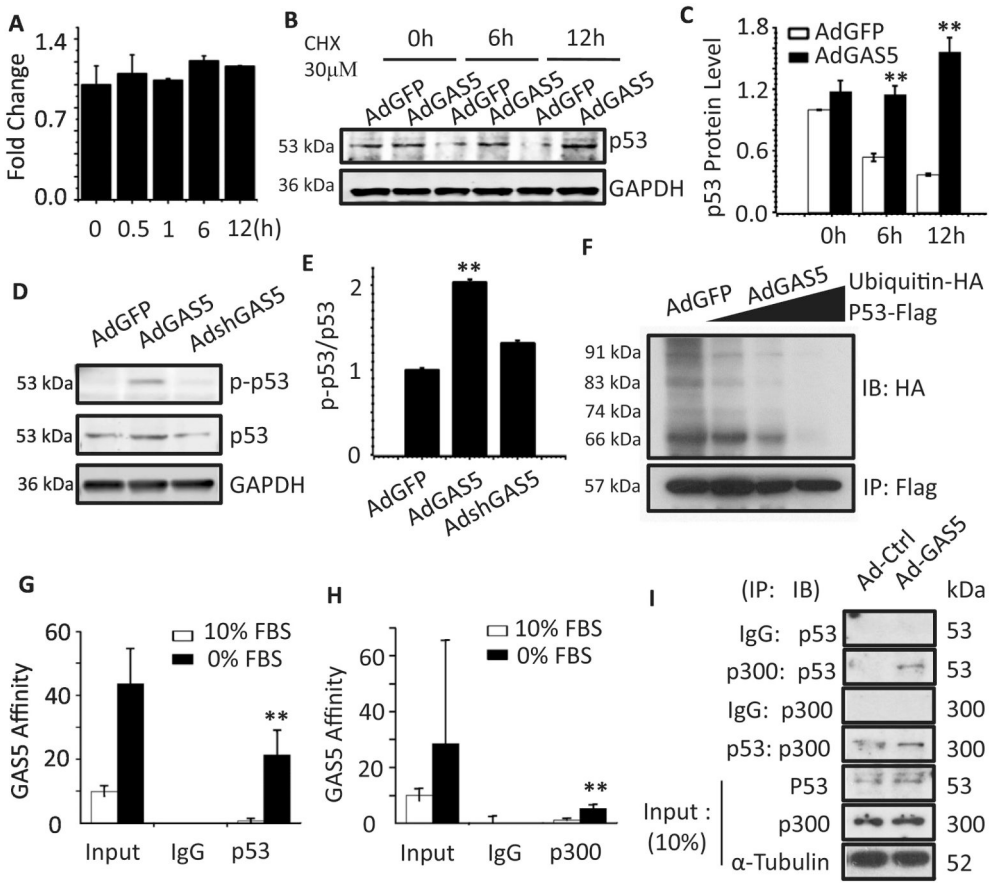

Figure 4: GAS5 enhanced p53 stability through recruiting p300 in VSMC.

A) GAS5 did not alter p53 gene transcription. VSMCs were transduced with AdGAS5 for 0 to 12 h, and p53 mRNA expression was detected by RT-qPCR. **B)** GAS5 protected p53 from degradation. VSMCs were transduced with AdGFP or AdGAS5 and then treated with 30 μM cycloheximide (CHX) for the time indicated. p53 protein levels were detected by Western blot. **C)** Quantification of p53 levels in B by normalizing to GAPDH. **D-E)** GAS5 promoted p53 phosphorylation. VSMCs were transduced with AdGFP, AdGAS5, or AdshGAS5. p53 phosphorylation was assessed by Western blot (D) and quantified (E) by normalizing to the total p53 level. **F)** GAS5 blocked p53 ubiquitination. VSMCs were transduced with AdGFP or different amount of AdGAS5 (1:1000, 1:500, 1:100) for 24 h then transfected with Ubiquitin-HA and p53-Flag plasmids. 24 h later, Flag-tagged p53 was pulled down, and p53 ubiquitination was detected by blotting the HA tag. **G)** GAS5 bound p53 as assessed by RNA immunoprecipitation assay. p53 in VSMCs was pulled down, and GAS5 was detected in the precipitates by RT-qPCR. Serum starvation (0% FBS) increased GAS5-p53 interaction. **H)** GAS5 bound p300 as assessed by RIP assay. Serum starvation increased GAS5-p300 interaction. **I)** Overexpression of GAS5 increased p300-p53 interaction as assessed by co-immunoprecipitation (co-IP) assay. * $p<0.05$; ** $p<0.01$; n=3.

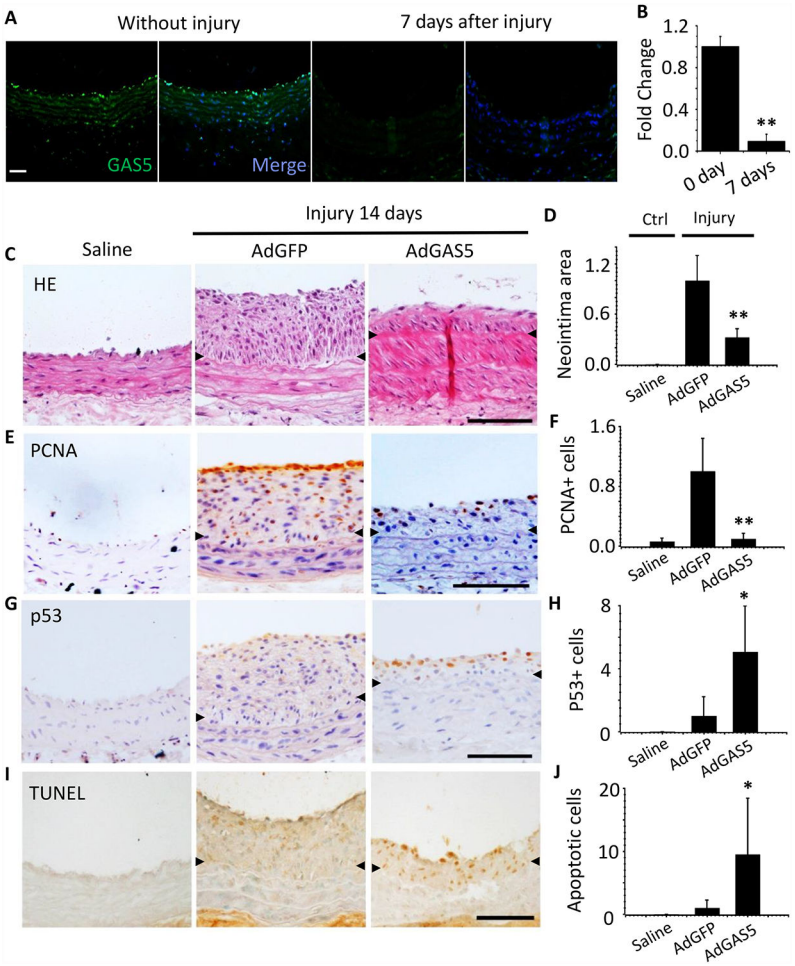

Figure 5: GAS5 suppressed injury-induced neointima formation.

A) GAS5 was expressed in normal arterial SMCs but was diminished in injured arteries. The rat carotid arteries without or with balloon injury for 7 days were used to detect GAS5 expression by RNA-FISH staining. Bar: 50 μm. **B)** GAS5 levels in carotid arteries were quantitatively analyzed by RT-qPCR. Relative mRNA expression was quantified by normalizing to Cyclophilin (CYP) mRNA. * $p<0.05$; ** $p<0.01$; n=5. **C)** Overexpression of GAS5 suppressed neointima formation. **D)** Quantification of neointima formation by averaging the neointima area in 10 different fields and shown as fold changes. **E)** Overexpression of GAS5 suppressed PCNA expression in neointimal VSMCs. **F)** Quantification of cell proliferation by averaging PCNA positive cells in 10 different fields and shown as fold changes. **G)** Overexpression of GAS5 increased p53 expression in neointima VSMC. **H)** Quantification of p53 positive cells in 10 different fields shown as fold changes. **I)** Overexpression of GAS5 increased neointima VSMC apoptosis as shown by TUNEL assay. **J)** Quantification of apoptotic cells in 10 different fields shown as fold changes. Boundary between neointima and media were marked with arrows. Bar: 100 μm. * $p<0.05$; ** $p<0.01$; n=5.

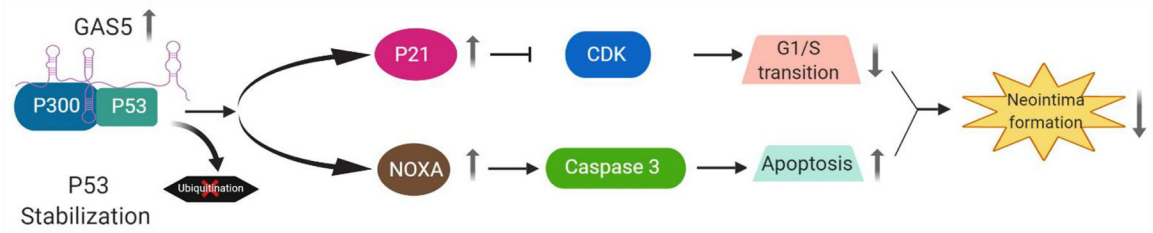

Figure 6: The mechanism whereby GAS5 regulates VSMC cell cycle and survival.

GAS5 binds p53 and its activator p300 to enhance p53 protein stability and its activity, causing increased expression of cell cycle regulators p21, which inhibits CDK1 activity, leading to cell cycle arrest. Increased p53 expression/activity also enhances NOXA expression, which promotes Caspase 3 cleavage and thus VSMC apoptosis. The combined effects of cell cycle arrest and apoptosis cause the reduction of injury-induced neointima formation.