Endothelium-specific overexpression of human *IC53* downregulates endothelial nitric oxide synthase activity and elevates systolic blood pressure in mice

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Aims Hypertension is one of the major risk factors for cardiovascular diseases. Endothelial cells (ECs) exert important functions in the regulation of blood pressure. A novel gene, *IC53*, as an isoform of the cyclin-dependent kinase (CDK)-binding protein gene *C53*, is mainly expressed in vascular ECs and is upregulated in the failing heart of rats. Overexpression of *IC53* promotes proliferation of ECs. To examine whether IC53 plays a role in the regulation of vascular tone and blood pressure, we constructed a transgenic (tg) mouse model of the *IC53* gene and studied its phenotypes relevant to vascular function.
Methods and results *IC53* cDNA was cloned from a human aorta cDNA library. Using the endothelium-specific VE-cadherin promoter, we constructed tg mice in which *IC53* was specifically overexpressed in vascular endothelia and found that the tg mice exhibit elevated systolic blood pressure (SBP) in contrast to the wild-type (wt) controls. Further studies revealed impaired endothelium-dependent vasodilation, reduced nitric oxide (NO) production and decreased endothelial NO synthase (eNOS) expression, and activity in the tg mice. Inhibition of IC53 in human umbilical vein ECs induces upregulation of eNOS activity.
Conclusion Our results indicate that *IC53* participates in the regulation of vascular homeostasis. Endothelium-specific overexpression of *IC53* is associated with elevated SBP, which may be in part attributed to the downregulation of eNOS signalling.

1. Introduction

Cardiovascular diseases are the main cause of mortality and morbidity in developed countries. Systolic blood pressure (SBP) is one of the most powerful predictors of coronary events and strokes.[1,2] Blood pressure is determined by multiple factors such as the cardiac output, circulating volume, and vascular wall resistance. Endothelial cells (ECs) lining the luminal surface of the blood vessels constitute a prime cellular component responsible for the regulation of blood pressure. By secreting various vasoactive molecules such as nitric oxide (NO) and endothelins, ECs modulate vascular contraction and dilation in response to diverse stimuli. In the case of endothelial dysfunction, the equilibrium between relaxation and contraction factors is disrupted and results in disturbances in the regulation of blood pressure.[3,4]

Recently, we cloned a novel gene named *IC53* (GenBank Accession No. AF110322) from a human aorta cDNA library. This gene was mapped to chromosome 17q21.31 with 12 exons, 2538 bp in cDNA length, encoding a putative protein of 419 amino acids, with the theoretical isoelectric point of 4.56 and calculated molecular mass of 46.3 kDa. Sequence comparison showed that this gene is an isoform of the *C53* gene which encodes a cyclin-dependent kinase

5 (CDK5)-binding protein.[5] It has been found that C53 participates in the regulation of cell-cycle checkpoint response and neuronal proliferation.[6–8] Our results showed that the *IC53* gene is mainly expressed in ECs and is upregulated in rat models of heart failure, and overexpression of *IC53* promotes proliferation of ECV304 cells.[9]

Nonetheless, the potential role of IC53 in endothelium-mediated blood pressure regulation is still unknown. Here, we constructed an endothelium-specific IC53 cDNA transgenic (tg) mouse and studied the *in vivo* parameters and related signal pathways. We found that IC53 overexpression diminished endothelium-dependent vasodilation while causing an elevation in SBP. These observations were associated with a downregulation of endothelial nitric oxide synthase (eNOS)/NO pathway.

2. Methods

All experiments were performed in accordance with the Declaration of Helsinki and were approved by the Committee for Animal Experiments of Chinese Academy of Medical Sciences and Peking Union Medical College (CAMS/PUMC).

2.1 Generation of tg mice

2.1.1 Plasmid construct
The PBSmVELacZ plasmid containing the mouse vascular endothelial cadherin promoter was obtained as a gift from Dr Kenneth Walsh, Boston University School of Medicine, Boston, MA, USA. The LacZ open-reading frame (ORF) fragment within the PBSmVELacZ plasmid was removed with *Xho*I/*Not*I (New England BioLabs, USA), replaced by the *IC53* cDNA, which was obtained from the pGEMIC53 plasmid with *Sal*I/*Not*I (New England BioLabs) and recovered from electrophoresis gel. The newly cloned PBSmVEIC53 plasmid is 6917 bp in length and contains an SV40 polyA downstream of the *IC53* cDNA (*Figure 1A*).

2.1.2 Generation and identification of tg mice
After digesting the PBSmVEIC53 plasmid with *Sal*I/*Bsp*HI, the 4.1 kb fragment was purified using agarose gel electrophoresis and DNA recovery kit (QIAGEN, Germany). The donor eggs from C57BL/6 mice (provided by the Experimental Animal Center of the CAMS/PUMC) were prepared for microinjection. The injected eggs were then transferred into the oviducts of pseudopregnant KunMing mice as foster mothers (anaesthetized by tribromoethanol saturated solution, 450 µL per mouse) and allowed to develop to term. Founder mice were identified by standard Southern blot analysis of tail DNA. A 605 bp DNA fragment amplified from the DNA for microinjection was used as the probe for Southern blot analysis. Primer sequences were sense: 5′-CTCCTGGTTCGGAATGTCA-3′; antisense: 5′-GCAACAGCATCGTCTCCC-3′. Six strains of founders were fertile and used to establish homozygous lines.

2.2 Phenotype analysis

2.2.1 *In vivo* measurements of blood pressure
Blood pressure of mice was recorded under both conscious and anaesthetized conditions. According to the manufacturer instructions, we used the computerized tail-cuff system, BA-98A system (Softron Co., Tokyo, Japan), with a photoelectric sensor to measure the blood pressure of conscious mice. We used sodium pentobarbital (90 mg/kg, i.p., 200 µL per mouse) to anaesthetize mice and fixed them on a heating pad (37°C). A catheter of stretched polyethylene tube filled with phosphate buffer solution (PBS) containing 50 U heparin/mL was inserted into the exposed right carotid artery; pulsatile blood pressure was recorded using a pressure converter (Institute of Aerospace Medicine, Beijing, P.R.

China) connected to a computer. In the pilot experiment, 100 µL saline was injected into the left jugular vein and no significant blood pressure alternation was detected. Solutions of the acetyl choline (ACh) and sodium nitroprusside (SNP) (Sigma Chemicals, USA) were prepared at the concentration of 0.1 nmol/mL, and the medicine was applied as a bolus (100 µL) through the left jugular vein. The N^G-nitro-L-arginine methyl ester (L-NAME) solution was injected intravenously at 50 mg/kg and blood pressure was recorded 30 min after application. Animals showing any sign of haemorrhage, arrhythmia, or low SBP (<60 mmHg) were excluded. The data were recorded and analysed with BL420E software (Chengdu Technology & Market Corp, P.R. China).

2.2.2 Echocardiographic evaluation
Cross-sectional, two-dimensional transthoracic echocardiography was performed by experienced sonographers using a VisualSonics imaging system (Toronto, Canada). A 30 mHz transducer was used for the studies. The chests of the mice were shaved and treated with a chemical hair remover to reduce ultrasound attenuation. Heart rate and core temperature were continuously monitored. Normothermic mouse core temperatures were maintained using a heated platform. Mice were anaesthetized with 1–2% isoflurane. Images were recorded and analysed in the ultrasound system. Ventricular function assessed from two-dimensional directed M-mode echocardiographic images was obtained from the parasternal short-axis view. All measurements were obtained in triplicate and averaged.

2.2.3 *Ex vivo* studies of aortic tone
Mice were sacrificed and the descending aorta was carefully removed, and a cylindrical segment (3 mm long) was excised from aorta. The rings were equilibrated in an organ bath for 30 min under a resting tension of 0.3 g in carbogenated (95% O_2/5% CO_2) Krebs bicarbonate solution, pH7.4 [in mmol/L, NaCl 120, KCl 5.2, $CaCl_2$ 2.4, $MgSO_4 7H_2O$ 1.2, $NaHCO_3$ 25, Na_2-EDTA 0.03, and dextrose (pH 7.4) 11]; the bath temperature was kept at 37°C. Subsequently, aortic ring contraction was induced with phenylephrine (PE) (Sigma Chemicals, USA), and the relaxation was induced with a cumulative dose of ACh or SNP. The relaxation responses were expressed as mean ± SEM in percentage values showing reversal of the PE-induced contractile responses.

2.3 Mechanism analysis

2.3.1 Preparation of tissues and serum
The fresh tissue (200 mg) was powdered in the presence of liquid nitrogen. The powdered samples were dissolved and subsequently homogenized at 4°C using a homogenizer in 1 mL buffer containing 0.14 mol/L NaCl, 2.6 mmol/L KCl, 8 mmol/L Na_2HPO_4, 1.4 mmol/L KH_2PO_4, 1% Triton X-100, pH 7.4. The homogenates were centrifuged at 10 000g at 4°C for 60 min. The supernatants were kept at -70°C for eNOS activity assay and protein expression analysis. The blood samples were collected and centrifuged at 14 000 r.p.m. for 15 min. The serum was kept for endothelin-1 (ET-1) and NO assays.

2.3.2 Reverse transcriptase—PCR
Total RNA from freshly harvested tissues was extracted using TRIZOL reagent (Invitrogen, USA). The reverse transcriptase–PCR was performed using the Primescript RT–PCR Kit (TAKARA, Japan) according to the manufacturer's instructions. Primer sequences were *IC53* sense: 5′-GGGAAACTCAACGGTGTACGAG-3′, antisense: 5′-TTCAAGCAGTGTCAGGGCAT–CT-3′; *GAPDH* sense: 5′-CACCCTGTTGCTGTAGCCGTATTC-3′, antisense: 5′-CAAGGCTGTGGGCAAGGTCATC-3′.

2.3.3 Fluorescent real-time quantitative PCR
Real-time PCR was performed with a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The total RNA from aorta was subjected to reverse transcriptase and real-time PCR
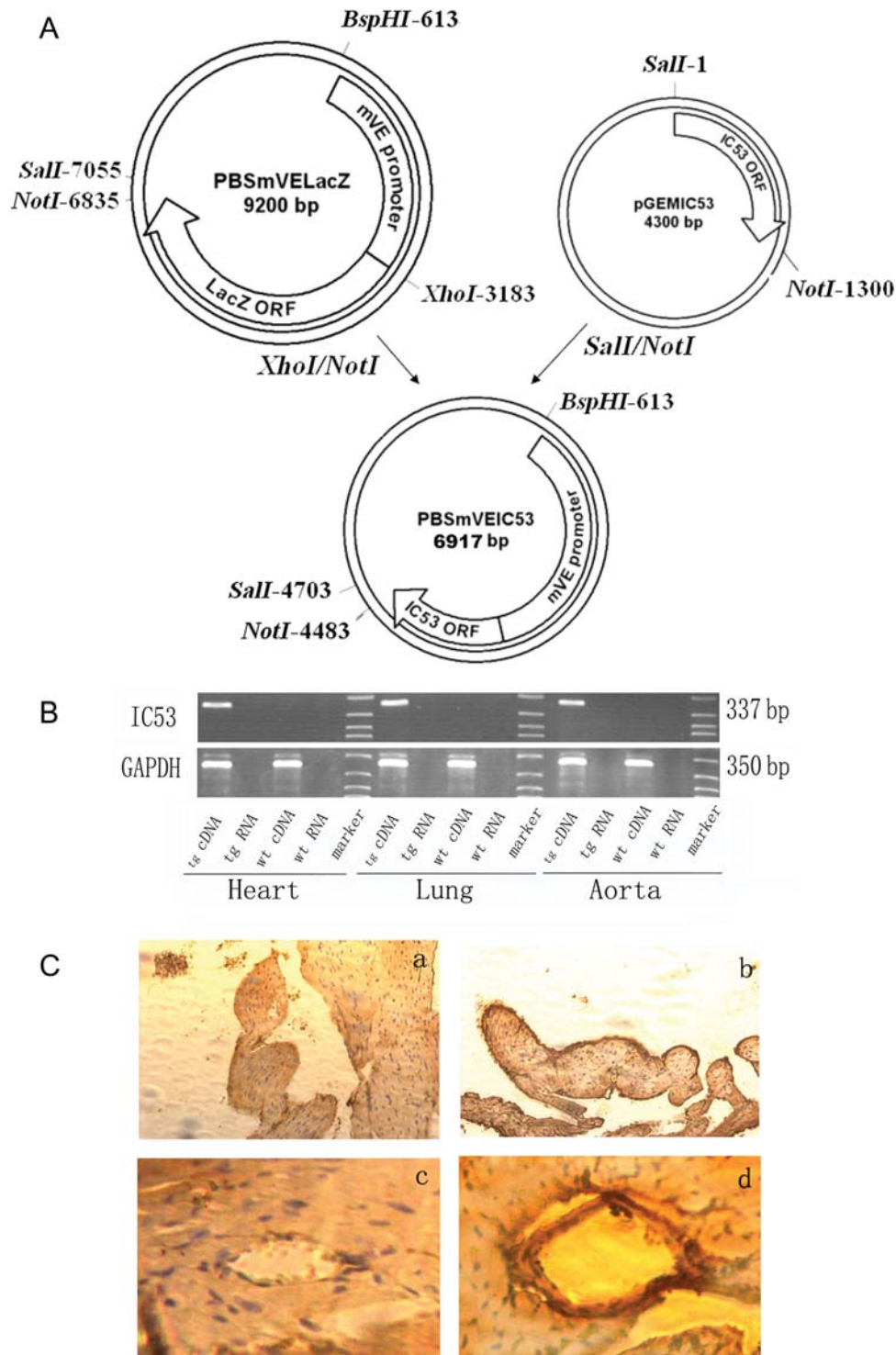
Figure 1 (*A*) The LacZ ORF of the PBSmVELacZ plasmid was replaced by the IC53 cDNA, which was isolated from the PGEMIC53 plasmid by digestion with *Sal*I/*Not*I. The newly cloned PBSmVEIC53 plasmid was digested with *Bsp*HI/*Sal*I; the 4.1 kb DNA fragment was recovered from electrophoresis gel and purified for microinjection. (*B*) Identification of the transcription of IC53 in tissue (top panels). The GAPDH is used as control for cDNA quality test (bottom panels). The DNA marker is DL2000 (TAKARA, Japan). (*C*) Localization of IC53 transcripts in the cardiac tissue sections. Positive staining signals were detected in the endothelium layer of endocardium (b) and small vessels (d) of tg mice but not in those of wt mice (a and c). Scale ×80 in a and b, ×320 in c and d.

with SYBR GREEN PCR Master Mix reagents (Applied Biosystems) and gene-specific primers designed on the basis of published nucleotide sequences. The *β-actin* gene is used as internal reference. Primers sequences were *eNOS* sense: 5′-ACAAATAGAGGCAATCTTCGTTCA-3′, antisense: 5′-CTATAGCCCGCATAGCGTATCA-3′; *β-actin* sense: 5′-TGCTGATCCACATCTGCTGG-3′, antisense: 5′-ATCATTGCTCCTCCTGAGCG-3′; mouse *IC53* sense 5′-TGTGTCCTCCACCCTCCTTC-3′, antisense

5′-GTGACCCTTCCTCATGGCTC-3′. The values were the mean of triplicate assays.

2.3.4 *In situ* hybridization
This experiment was conducted using the *in situ* hybridization kit (DingGuo Biotechnology Company Ltd, Beijing, China). A 300 bp

fragment from the human *IC53* cDNA was used as template for the synthesis of oligonucleotides DNA probe, which was subsequently labelled with digoxigenin (DIG). Paraffin-embedded sections were hybridized with probes and processed using standard procedures with anti-DIG antibody-conjugated alkaline phosphatase.

2.3.5 Human umbilical vein endothelial cell cultivation and small hairpin RNA inhibition

Human umbilical vein endothelial cells (HUVECs) were freshly isolated using the collagenase IV method as described previously[10] and cultured in medium M200 (Cascade Biologics Inc.). Cells between third and sixth passages were studied. The IC53 siRNA (GGAATCAGATTCAAAGGAT) was designed using the BLOCK-iT RNAi designer and incorporated into pSIREN-RetroQ vector (Clontech) to generate pSIREN-RetroQ-IC53 expressing C53 siRNA. The sequences were built into duplex oligonucleotides using the design tool at the Clontech website. The construct contained the following elements: a *Bam*HI cloning site at the 5′ end, the coding strand sequence of the hairpin (as above), a loop region, the complementary non-coding strand sequence of the hairpin, an RNA polymerase III termination sequence, and a 3′ *Eco*RI cloning site. Retrovirus stocks were prepared by co-transfecting 293T cells (Clontech) with plasmid pMD (coding for murine leukaemia virus gag/pol) together with pVSV-G and pSIREN-RetroQ plasmid. Cell supernatants were harvested 72 h after transfection, clarified, filtered, and stored as recommended by the supplier. Target cells were infected with retrovirus supernatants, and then cells with integrated viral sequences were selected using puromycin at 2 µg/mL and then passaged with continuous puromycin selection. The degree of IC53 silencing was regularly monitored by real-time PCR. Control cells were infected with the control retroviruses (pSIREN-RetroQ SiGFP) in parallel.

2.3.6 Assay of NO concentrations and eNOS activity

Serum NO concentrations and aortic eNOS activity were determined using the NO assay kit and eNOS assay kit (Catalogue No. A012 and A014, Jiancheng Bioengineering Institute, Nanjing, P.R. China, www.njjcbio.com) according to the manufacturer's protocol. The activity of eNOS was assayed by nitrites and nitrates produced from L-arginine. In brief, the supernatants of aorta tissue homogenates (100 µL) were incubated in 50 mmol/L Tris–HCl buffer (pH 7.5) containing the co-factors and the substrate L-arginine, for 15 min at 37°C. After the incubation period, the reaction was quenched by the addition of 1 mL of stop buffer. The concentration of the nitrites and nitrates in the reaction mixture was determined by colorimetric method (530 nm) to evaluate the eNOS activity. The protein concentration was determined by the BCA method. All samples were assayed in triplicate and averaged.

2.3.7 Determination of serum ET-1 concentrations

Measuring of ET-1 concentrations in serum was conducted using the radioimmunoassay kit (The Radioimmunology Institute of the Chinese People Liberation Army General Hospital, Beijing, P.R. China) according to the manufacturer's illustration. Samples in triplicate were assayed and the values were averaged.

2.3.8 Determination of intracellular cAMP and PKA in HUVEC

The HUVECs were harvested and lysis reagent added in the commercial kit (Amersham Pharmacia Biotech). Intracellular cAMP concentration in HUVEC was measured by the commercial kit using the non-acetylation procedure. Samples were standardized for total cellular protein by the Bradford method and cAMP concentration calculated as fmol per microgram protein. The activities of PKA were measured by the protein kinase assay kit (Uscnlife company, USA). The kit is based on ELISA, which utilizes a synthetic peptide and a monoclonal antibody recognizing the PKA peptide.

2.3.9 Western blot analysis

Fresh aortic tissue was homogenized in lysis buffer [in mmol/L, NaCl 150, Tris (pH 7.5) 30, and PMSF 1; 0.25 mol/L sucrose; 5 µg/mL leueptin; and 1.9 µg/mL aprotinin]. Sixty micrograms of proteins were separated by SDS–PAGE and probed with antibodies against eNOS and β-actin (Catalogue No. sc-1506 and sc-47778, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Signals were revealed with chemiluminescence and visualized by autoradiography. Samples in triplicate were assayed and the values were averaged.

2.4 Statistics

Results are presented as mean ± SEM. Comparisons between groups were made by paired *t*-test or ANOVA. A *P*-value <0.05 was considered statistically significant.

3. Results

3.1 Identification of endothelium-specific tg mice

3.1.1 Generation of the IC53 tg mice

The human *IC53* cDNA was cloned downstream of the mouse vascular endothelial cadherin promoter, which directs endothelium-targeted transcription (*Figure 1A*).[11] Purified linear DNA was microinjected into fertilized eggs of C57BL6, which were transferred into oviducts of foster mice. Six strains of tg mice (F0 generation) were identified from 22 offspring using Southern blot. Homozygous offspring of one strain (F1 generation) were used for further investigation.

3.1.2 Endothelium-targeted transcription of *IC53*

Transcription of *IC53* was identified using the reverse transcriptase–PCR analysis (*Figure 1B*). Total RNA was extracted from the heart, lung, and aorta tissues. Primers were designed according to the specific sequence of the transgene. A band of 337 bp was identified from the cDNA templates of the tg mice but not from the wild-type (wt) controls. Sequencing analysis confirmed that the resultant PCR products are identical to the transgene cDNA. To exclude the possibility of genomic DNA contamination, total RNA samples were also used as templates for PCR amplification, but no bands identical to the transgene *cDNA* were found.

Transcription of human *IC53* was confirmed by the *in situ* hybridization method. Positive staining was identified only in the endothelium of various organs in the tg mice but not in those of the wt mice (*Figure 1C*). These results confirmed that transcripts of *IC53* were specifically expressed in the endothelium of the tg mice.

3.2 Phenotype analysis

3.2.1 The haemodynamic parameters *in vivo*

No apparent difference was found in the appearance or performance between the tg mice and wt controls. At the age of 16–18 weeks, the male offspring were subjected to haemodynamics analysis, including SBP, diastolic blood pressure (DBP), mean artery pressure (MAP), and pulse pressure (PP). At conscious situation, the tg mice showed higher SBP than their wt littermates (124.61 ± 2.34 vs. 108.71 ± 3.07 mmHg, *P* < 0.05); the MAP were 104.22 ± 2.98 vs. 95.39 ± 2.02 mmHg; the DBP were 85.90 ± 3.43 vs. 77.85 ± 2.51 mmHg; the PP were 37.64 ± 2.98 vs. 32.30 ± 2.85 mmHg (*Figure 2A*). Similar trends were found in anaesthetized animals, the SBP were 105.32 ± 3.66 vs. 90.53 ± 2.74 mmHg (*P* < 0.05), the MAP, DBP, and PP were
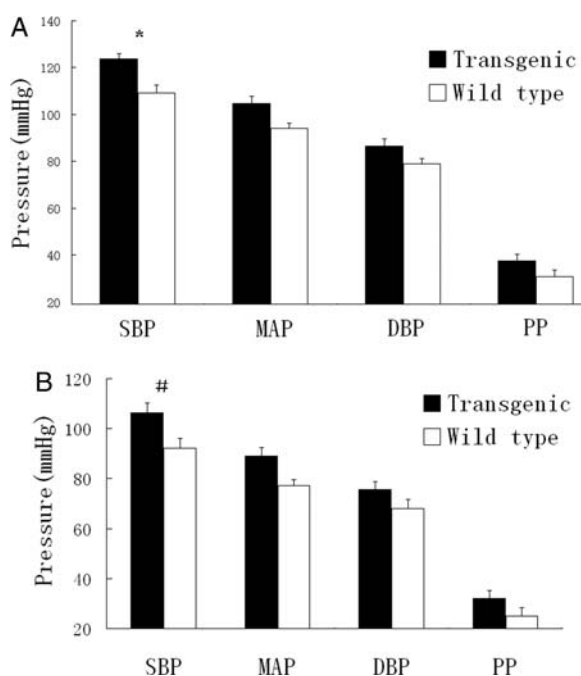
Figure 2 Haemodynamic parameters, including the systolic blood pressure (SBP), diastolic blood pressure (DBP), mean artery pressure (MAP), and pulse pressure (PP), are recorded. (*A*) Results of the conscious mice. $n = 14$ per group; *$P < 0.05$ vs. wt. (*B*) Results of the anaesthetized mice. $n = 12$ per group; #$P < 0.05$ vs. wt.

also higher in the tg mice than those in the wt controls (*Figure 2B*).

Measurements of cardiac output in the anaesthetized mice were carried out by cardiac ECHO analysis. No significant difference was detected in the cardiac output between the tg mice and wt mice (22.29 $\pm$ 1.83 vs. 21.02 $\pm$ 1.08 mL/min, $n = 14$, $P = 0.21$).

3.2.2 Pressure response *in vivo*
Three kinds of vasoactive medicines, ACh, SNP, and L-NAME, were employed and responses of blood pressures were analysed.

Blood pressure dropped immediately in response to the application of vasodilators (*Table 1*). The decrease percentage in MAP induced by ACh in tg group was much lower than that in wt groups (decrease level in percentage: 27.8 vs. 51.2, $P < 0.05$). However, both groups had similar response when SNP was applied (28.6 vs. 30.8%).

In the presence of L-NAME, MAP increased from 95.13 $\pm$ 2.72 mmHg to 121.36 $\pm$ 2.89 mmHg in the wt mice, whereas the tg mice had an MAP increase from 104.46 $\pm$ 2.41 mmHg to 115.57 $\pm$ 2.55 mmHg; the changes in MAP

were 26.17 $\pm$ 2.01 vs. 11.14 $\pm$ 2.31 mmHg for wt vs. tg (27.51 vs. 10.71%; $n = 12$, $P < 0.05$), respectively.

3.2.3 Vasodilation *ex vivo*
The aortic rings were sensitive to ACh and SNP, and the relaxation started from the concentration of 10^{-8} mol/L of the vasodilators (*Figure 3*). EC50, the negative logarithm of the concentration of vasodilator causing 50% relaxation of the aortic strips, was calculated. The EC50 for ACh was apparently higher in tg mice when compared with wt mice (7.21 $\pm$ 0.03 vs. 6.85 $\pm$ 0.02; $n = 12$; $P < 0.05$). But the EC50 for SNP in tg mice was similar to that in wt mice (6.92 $\pm$ 0.05 vs. 6.98 $\pm$ 0.04; $n = 12$).

3.3 Molecular analysis

3.3.1 Serum NO and ET-1 levels
There is no significant difference in the serum ET-1 concentration between two groups (63.97 $\pm$ 7.38 vs. 61.37 $\pm$ 4.96 pg/mL; $n = 6$) (*Figure 4A*), whereas the serum NO level decreased dramatically in tg mice in contrast to their wt littermates (26.42 $\pm$ 4.43 vs. 43.36 $\pm$ 8.65 μmol/L; $n = 6$, $P < 0.05$) (*Figure 4B*).

3.3.2 The eNOS activities in aorta
The eNOS activity in aorta was markedly decreased in the tg mice compared with wt mice (0.74 $\pm$ 0.16 vs. 1.90 $\pm$ 0.11 U/mg protein; $n = 6$, $P < 0.05$) (*Figure 4C*).

3.3.3 The mRNA and protein levels of eNOS in aorta
The expression of eNOS in aorta was examined using the real-time quantitative PCR and western blot. Whereas no significant change was detected in the mRNA level (relative to β-actin, 1.01 $\pm$ 0.14 vs. 0.90 $\pm$ 0.09, $n = 6$) (*Figure 4D*), the eNOS protein expression in tg mice was significantly reduced comparing with that in wt controls (relative to β-actin, 0.37 $\pm$ 0.06 vs. 0.91 $\pm$ 0.08, $n = 6$, $P < 0.05$) (*Figure 4E* and *F*).

3.3.4 The mRNA levels of mouse endogenous *IC53* gene in aorta
Using real-time PCR method, we further analysed the transcription of mouse endogenous *IC53* gene and found that the mRNA level of mouse *IC53* gene in aorta decreased significantly in comparison with that of wt controls (relative to β-actin, 0.36 $\pm$ 0.04 vs. 1.00 $\pm$ 0.16, $n = 6$, $P < 0.05$) (*Figure 4G*).

3.3.5 Cell culture
The real-time analysis revealed that the endogenous *IC53* gene mRNA reduced 78.5% by small hairpin RNA (shRNA)

Table 1 The decreases in MAP induced by ACh and SNP in mice

BP	Baseline	SNP added		ACh added	
		BP level	Decrease per cent	BP level	Decrease per cent
wt	76.25 $\pm$ 2.43	54.37 $\pm$ 3.49	28.63 $\pm$ 5.19	37.87 $\pm$ 2.29	51.22 $\pm$ 3.75
tg	86.50 $\pm$ 3.53	59.75 $\pm$ 3.69	30.83 $\pm$ 5.07	62.85 $\pm$ 2.91	27.82 $\pm$ 3.61*

It was the maximal decrease in MAP that was measured and analysed.
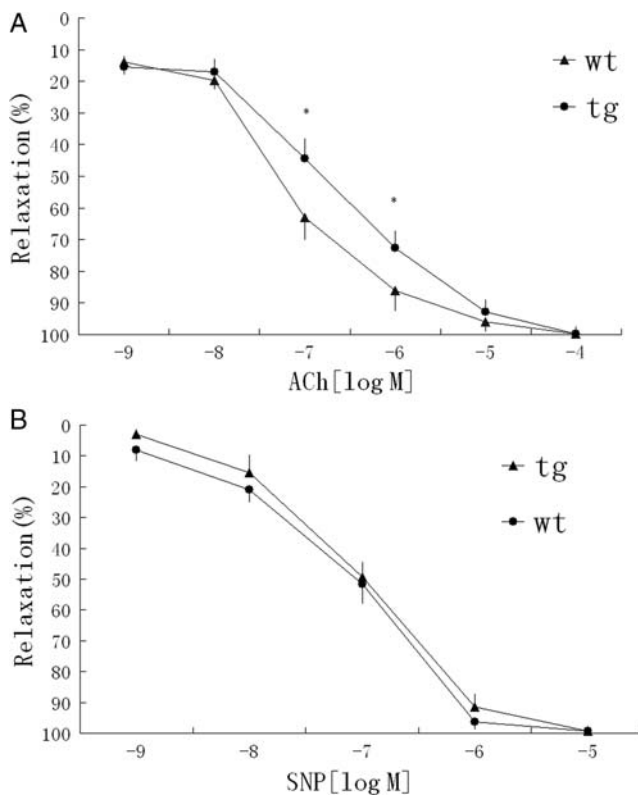*$P < 0.05$ compared with wt, $n = 14$ per group.

Figure 3 (*A*) The dose–response curves of aortic rings to ACh in tg and wt mice. (*B*) The dose–response curves of aortic rings to SNP in mice. Magnitudes of relaxation are mean $\pm$ SEM of percentage reversal of PE-induced contractile responses. $n = 12$ in 12 mice per group. *$P < 0.05$ vs. wt.

inhibition in comparison with control. The eNOS mRNA level did not change but the enzyme activity increased 126.3% in comparison with control (*Figure 4H*).

We further analysed the cAMP levels and PKA activities in HUVEC, but no significant difference was found between shRNA and control groups (12.1 vs. 13.0 fmol/μg protein, 0.43 vs. 0.39 pmol/min μg protein, respectively, $P > 0.05$).

4. Discussion

In the current study, we explored a potential role of the *IC53* gene in endothelium-dependent blood pressure regulation. We created tg mice in which the human *IC53* gene is over-expressed specifically in vascular ECs. The tg mice showed elevated SBP and decreased eNOS activity comparing with wt control.

Previous studies have established that SBP elevation is associated with decreased arterial compliance, which is inducible by endothelial dysfunction that is characterized by decreases in NO bioavailability and impairs endothelium-dependent vasodilation.[12–15] Restoration of EC function will improve arterial compliance and lower blood pressure.[16–18]

Here, we studied the vasodilation functions of the mice both *in vivo* and *ex vivo*. ACh is a kind of endothelium-dependent NO producer, whereas SNP can produce NO independently. In accordance with previous findings, our results have shown that vascular reactions to SNP were not changed, but reaction to ACh was significantly attenuated in tg mice, indicating that the endothelium-mediated vasodilation was impaired. Thus, we hypothesize that in our

current research, the elevated SBP of tg mice is caused by endothelial dysfunction in vasodilation.

ECs modulate vascular tone by secreting various active factors, among which NO and ET-1 are the two important regulators. We found that serum ET-1 level was not changed, whereas NO level decreased dramatically in tg mice. Since NO is responsible for the basic vasotonic regulation, a diminished NO level will hamper relaxation of the vascular smooth muscles, resulting in greater rigidity of the artery wall and SBP elevation.[19–22] Endogenous NO is mainly produced by eNOS, which is constitutively expressed in the vascular endothelium throughout the cardiovascular system, playing a major role in the regulation of blood pressure and vascular tone.[23–25] Inhibition of eNOS leads to blood pressure elevation in animals and humans.[25–27]

In the IC53 tg mice, SBP elevation was associated with decreases in the NO concentration, eNOS protein level, and enzyme activity. In HUVECs, inhibition of IC53 upregulates eNOS activity. In addition, L-NAME, a non-specific NOS inhibitor increased blood pressure dramatically in wt mice but marginally in tg mice, suggesting that NO bioavailability was reduced in tg mice. On the basis of the above data, we suggest that a loss of NO consequent to the down-regulation of eNOS underlies endothelial dysfunction and SBP elevation in tg mice.

Our data also share similarities with previous reports that knockout of the *eNOS* gene induced elevated blood pressure.[28] The increased amplitude in blood pressure of *IC53* tg mice was not as robust as that of the $eNOS^{-/-}$ mice, probably because the *IC53* tg mice still preserve part of eNOS activity (about 50% of normal). The BP levels of our animals, including the controls, were slightly lower in comparison with previous reports,[29] in which SBP of wt mice was mostly >110 mmHg. In each group of our experiments, 14 mice were analysed and the data were highly consistent. Therefore, we thought that this discrepancy could be due to the differences in mouse strain, gender, and anaesthetic extent, as these factors have important impacts on blood pressure.

Schrader and colleagues previously reported that the coronary artery, unlike the aorta, was unaffected in its response to ACh in $eNOS^{-/-}$ mice; they suggested that important compensatory mechanisms are activated in the coronary artery of $eNOS^{-/-}$ mice.[30] It would be interesting to study the coronary relaxation of IC53 tg mice in our future experiments.

We found that the eNOS protein level was reduced in IC53 tg mice, which is consistent with the decreased eNOS activity. But there is no significant change in the *eNOS* mRNA level, suggesting that IC53 modulates *eNOS* expression at the translational or post-translational level. We also discovered that mouse endogenous *IC53* transcription was inhibited in tg mice; there might be an antagonizing relation between these two alleles.

In summary, endothelium-specific overexpression of *IC53* gene resulted in elevated SBP and impairment in endothelium-dependent vasodilation, and this could be caused by eNOS downregulation. Thus, we have established an innovative role of IC53 in modulating endothelial function via the eNOS pathway. These findings will promote further studies investigating potential roles of IC53 in the pathogenesis of hypertension and other cardiovascular diseases associated with endothelial functions.
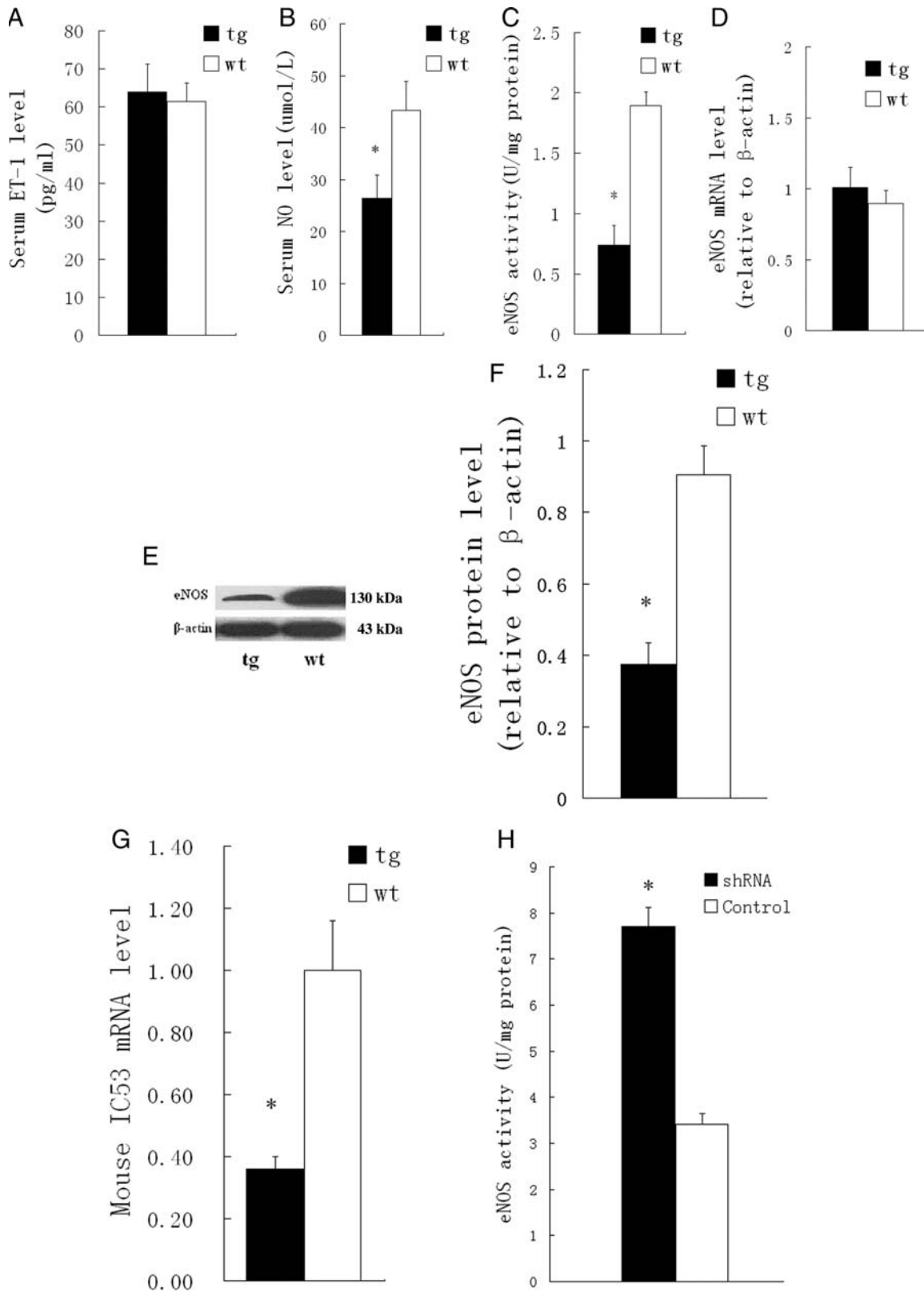
Figure 4 (*A*) Serum ET-1 concentrations in tg and wt mice. (*B*) Serum NO concentrations in tg and wt mice. (*C*) Aortic eNOS activities in tg and wt mice. One unit of the eNOS activity is defined as the ability to create 1 nmol NO within 1 min by 1 mg tissue protein. (*D*) Aortic eNOS mRNA levels in tg and wt mice. The values are the relative expression levels of samples comparing with β-actin. (*E*) Aortic eNOS protein levels in tg and wt mice, with molecular weight marked (KDa). (*F*) Quantification of the eNOS protein levels (relative to β-actin) by measuring the density of the bands in (E). (*G*) Endogenous IC53 mRNA levels in tg and wt mice aorta, relative to β-actin. (*H*) Intracellular eNOS activity in shRNA-treated and control HUVECs. $n = 6$ per group; *$P < 0.05$ vs. wt.

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