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ORIGINAL ARTICLE

ANTXR2 is a potential causative gene in the genome-wide association study of the blood pressure locus 4q21

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Hypertension is the most prevalent cardiovascular disease worldwide, but its genetic basis is poorly understood. Recently, genome-wide association studies identified 33 genetic loci that are associated with blood pressure. However, it has been difficult to determine whether these loci are causative owing to the lack of functional analyses. Of these 33 genome-wide association studies (GWAS) loci, the 4q21 locus, known as the fibroblast growth factor 5 (*FGF5*) locus, has been linked to blood pressure in Asians and Europeans. Using a mouse model, we aimed to identify a causative gene in the 4q21 locus, in which four genes (anthrax toxin receptor 2 (*ANTXR2*), PR domain-containing 8 (*PRDM8*), *FGF5* and chromosome 4 open reading frame 22 (*C4orf22*)) were near the lead single-nucleotide polymorphism (rs16998073). Initially, we examined *Fgf5* gene by measuring blood pressure in *Fgf5*-knockout mice. However, blood pressure did not differ between *Fgf5* knockout and wild-type mice. Therefore, the other candidate genes were studied by *in vivo* small interfering RNA (siRNA) silencing in mice. *Antxr2* siRNA was pretreated with polyethylenimine and injected into mouse tail veins, causing a significant decrease in *Antxr2* mRNA by 22% in the heart. Moreover, blood pressure measured under anesthesia in *Antxr2* siRNA-injected mice rose significantly compared with that of the controls. These results suggest that *ANTXR2* is a causative gene in the human 4q21 GWAS-blood pressure locus. Additional functional studies of *ANTXR2* in blood pressure may identify a novel genetic pathway, thus increasing our understanding of the etiology of essential hypertension.
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Keywords: *ANTXR2*; blood pressure; causative gene; GWAS; siRNA *in vivo* delivery

INTRODUCTION

Hypertension is the most prevalent cardiovascular disease worldwide. Despite advances in our understanding of hypertension, it remains one of the greatest global public health problems.[1] Approximately 90–95% of hypertensive patients have essential hypertension, which is defined as having no identifiable cause.[2] Although genetic risk factors have been linked to the development of essential hypertension, its genetic basis is poorly understood.[3]

Recently, several genome-wide association studies (GWASs), including those by the Korean Association REsource (KARE),[4] Global Blood Pressure Genetics (Global BPgen),[5] Cohorts for Heart and Aging Research in Genome Epidemiology (CHARGE),[6] Asian Genetic Epidemiology Network for Blood Pressure (AGEN-BP),[7] and the International Consortium of Blood Pressure (ICBP),[8] identified 33 genetic loci that are associated with blood pressure. Of these loci, 4q21, known as the fibroblast growth factor 5 (*FGF5*) locus, had one of the strongest association signals with blood pressure in Asians and Europeans, reported as follows: Global BPgen ($P=1\times10^{-21}$ with diastolic blood pressure (DBP)),[5] Millennium Genome Project ($P=1.6\times10^{-8}$ with systolic blood pressur (SBP), $P=1.8\times10^{-7}$ with DBP),[9] AGEN ($P=3.9\times10^{-13}$ with SBP, $P=2.0\times10^{-11}$ with DBP)[7] and ICBP ($P=1.5\times10^{-23}$ with SBP, $P=8.5\times10^{-25}$ with DBP)[8] (Supplementary Table S1).

Four genes—anthrax toxin receptor 2 (*ANTXR2*), PR domain-containing 8 (*PRDM8*), *FGF5*, and chromosome 4 open reading frame 22 (*C4orf22*)—reside near the lead single-nucleotide polymorphism (rs16998073) of the 4q21 locus. *ANTXR2* was originally identified as being upregulated during capillary morphogenesis of the endothelium, thus implicating it in angiogenesis.[10]

FGF5 mediates several processes, including cell growth and morphogenesis.[11] *Fgf5*-knockout mice have extremely long hair, but blood pressure has not been examined in them.[12] *PRDM8* contains classical C2H2-type zinc finger domains, suggesting that it is involved in gene regulation,[13] and the function of *C4orf22* is unknown.

It has been difficult to identify the causative genes in most GWAS loci owing to a lack of functional analyses. Causative genes in GWAS loci must be determined to translate the results of GWASs into a greater understanding of blood pressure regulation and accelerate the

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development of advanced therapeutics. In this study, we aimed to identify a causative gene in the 4q21 locus using knockout mice and *in vivo* small interfering RNA (siRNA) silencing. The effects of *Fgf5* on blood pressure were examined in knockout mice, and *Antxr2*, *Prdm8* and *C4orf22* were studied by *in vivo* siRNA silencing in mice.

MATERIALS AND METHODS

Animals

B6.C-*Fgf5*^{go}/J mice, in which exon 1 of *Fgf5* is deleted, were purchased from The Jackson Laboratory (Bar Harbor, ME, USA).[12] Heterozygote B6.C-*Fgf5*^{go}/J mice were crossbred in a pathogen-free facility. Blood pressure measurements were taken in ~8-week-old B6.C-*Fgf5*^{go}/J mice.

BALB/c mice (Japan SLC, Shizuoka, Japan) were used at age 7–9 weeks for siRNA injection experiments and blood pressure measurements. Every effort was made to minimize the number of animals that were used and their suffering as per the Committee for the Care and Use of Laboratory Animals, the College of Pharmacy, Kyung Hee University (KHP-2010-04-06).

Genotyping of B6.C-*Fgf5*^{go}/J mice

Genomic DNA from an ear-punch slice was extracted using the Phire Animal Tissue Direct PCR Kit as per the manufacturer's instructions (Finnzyme, Vantaa, Finland). Briefly, 20 µl of dilution buffer and 0.5 µl of DNA release solution were mixed and added to a piece of mouse ear tissue (~2 mm in diameter). The tissue was incubated for 5 min at room temperature and placed into a preheated (98 °C) block for 2 min.

Based on the deleted region in B6.C-*Fgf5*^{go}/J mice, two pairs of PCR primers, Fgf5-Out and Fgf5-In, were used to genotype the progeny of B6.C-*Fgf5*^{go}/J mice (Figure 1b, Table 1). Fgf5-Out primers recognize the sequences outside of the deleted region, and Fgf5-In primers recognize sequences in the deleted region. The expected amplicon of the Fgf5-Out primer was 4.5 kb, which was too large for standard PCR amplification. Thus, the Fgf5-Out primer set generated a product only from the mutant allele (~2.9 kb) (Figure 1c).

The PCR program was as follows: 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 63.7 °C for 30 s and 72 °C for 1 min 30 s; and elongation at 72 °C for 10 min.

In vitro siRNA silencing in NIH3T3 cells

Three siRNAs were synthesized for each gene, and their efficacy was examined in NIH3T3 cells (Genolution, Seoul, Korea). The siRNAs were transfected into cells with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA); 1–2 µl of Lipofectamine 2000 and 20 nM siRNA were added to 50 µl phosphate-buffered saline, and the reaction was incubated for 10 min at room temperature. NIH3T3 cells were used to seed 24-well plates at 5×10^4 cells per well and 30% confluence, and the transfection mixture was added to each well. The cells were harvested to extract RNA after 24 h of incubation.

Total RNA was prepared using TRIzol reagent (Invitrogen) as per the manufacturer's instructions. The negative-control siRNA was selected from randomized sequences and did not match any sequence in GenBank. Table 2 shows the sequences of the *Antxr2*, *Prdm8* and negative-control siRNAs. Although we used several different primer sets for *C4orf22*, multiple bands were generated consistently by quantitative real-time PCR from NIH3T3 cells, and therefore, *C4orf22* was not examined further.

In vivo siRNA silencing in mice

The polyethylenimine transfection agent was *in vivo*-jetPEI (Polyplus, Illkirch-Graffenstaden, France). As per the instructions, 50 µg siRNA and 6.5 µl *in vivo*-jetPEI solution (N/P charge ratio of 6) were diluted with 50 µl 10% glucose solution and 50 µl sterile H_2O. The solution was vortexed gently and left for 15 min at room temperature. The mixture was injected into the mouse tail vein, and the injected mice were killed for RNA extraction.

RNA was extracted from mouse tissues with TRIzol (Invitrogen) 24 h after the injection. We synthesized complementary DNA from 500 ng of total RNA using the PrimeScript RT kit (TaKaRa, Otsu, Japan) as per the manufacturer's instructions. The mRNA levels were analyzed by quantitative real-time PCR. One-tenth of the complementary DNA reaction was diluted to a final volume of 20 µl per reaction, containing SYBR Green I (TaKaRa). The quantitative real-time PCR was performed on an ABI Step One Real-time PCR system (Applied Biosystems, Foster, CA, USA) using the following program: 45 cycles of 95 °C for 10 s, 60 °C for 15 s and 72 °C for 20 s. Table 1 lists the primer sequences.

To normalize the amounts of sample complementary DNA in each real-time PCR reaction, the cycle threshold (Ct) value of Glyceraldehyde 3-phosphate dehydrogenase was subtracted from that of *Antxr2* to obtain the delta Ct value of *Antxr2*. To calculate the fold change in *Antxr2* mRNA expression, the delta Ct value of the control sample in a control siRNA-injected mouse was subtracted from that of the case sample in an *Antxr2* siRNA-injected mouse to obtain delta delta Ct (ΔΔCt). Relative levels of gene expression (that is, fold change) were determined using the $2^{-\Delta\Delta Ct}$ method.

Western blot

Total proteins were extracted from mouse tissue using PRO-PREP protein extraction solution (Intron Biotechnology, Gyeoggi-Do, Korea) as per the manufacturer's instructions. Protein concentrations were measured by Bradford assay.[14] Total proteins were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel and transferred to a nitrocellulose membrane (Pall, Ann Arbor, MI, USA). The membrane was blocked in 5% skimmed milk overnight at 4 °C and incubated with anti-actin (cat. no. sc-1616, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-Antxr2 (cat. no. AF3636, R&D Systems, Minneapolis, MN, USA) (both 1:1000) for 40 h at 4 °C.

For the secondary reaction, anti-goat immunoglobulin G-horseradish peroxidase (IgG-HRP, for actin) and anti-goat IgG-HRP (for Antxr2) (cat. no. sc-2020, Santa Cruz Biotechnology) were incubated with the blot for 2 h at room temperature. Protein signals were detected using Luminol (Santa Cruz Biotechnology), followed by exposure to X-ray films (Agfa-Health Care NV, Mortsel, Belgium).

Blood pressure

Blood pressure was recorded intra-arterially at the carotid artery with a computerized-data acquisition system (AD Instruments, Bella Vista, Australia). To place the intra-arterial catheter, mice were anesthetized with a standard dose of $0.018\,ml\,g^{-1}$ avertin, diluted 40-fold from the stock solution (10 g 2,2,2,-tribromoethanol dissolved in 10 ml tertiary amyl alcohol) in 0.9% NaCl. The polyethylene tube (0.2 mm ID, 0.5 mm OD) (Natsume, Tokyo, Japan) of the intra-arterial catheter was filled with $100\,U\,ml^{-1}$ heparin in 0.9% NaCl, inserted into the right-carotid artery, and tied in place.

The blood pressure in the vessel was transmitted along the catheter to the transducer's diaphragm (MLT0699 Disposable BP Transducer. AD Instruments). The diaphragm signal was amplified through a bridge amplifier and recorded on a PowerLab system (LabChart 7.3.5, AD Instruments). Blood pressure was monitored for up to 2 h after injection of the anesthetic and calculated as the average blood pressure between 40 min and 80 min (1 measure per min).[15] In siRNA-injected mice, blood pressure was measured 24 h after the siRNA injection.

Statistical analysis

The mRNA levels in *Antxr2* siRNA-injected mice are expressed as fold change ± standard error of the mean (s.e.m.) versus control mice. Differences in *Antxr2* mRNA and protein expression between case and control mice were analyzed using the Mann–Whitney *U*-test. Blood pressure is expressed as the mean ± s.e.m. and was analyzed by the Mann–Whitney *U*-test. Differences in blood pressure between genotypes of *Fgf5*-knockout mice were analyzed using the Kruskal–Wallis test. For all analyses, differences were considered significant at $P < 0.05$.

RESULTS

Blood pressure in B6.C-*Fgf5*^{go}/J mice

Figure 1a shows four genes (*Antxr2*, *Prdm8*, *Fgf5* and *1700007G11Rik*) in the murine 5qE3 locus, equivalent to the human 4q21 locus. The lead single-nucleotide polymorphism (rs16998073) in 4q21 is proximal and upstream of *FGF5*. Thus,

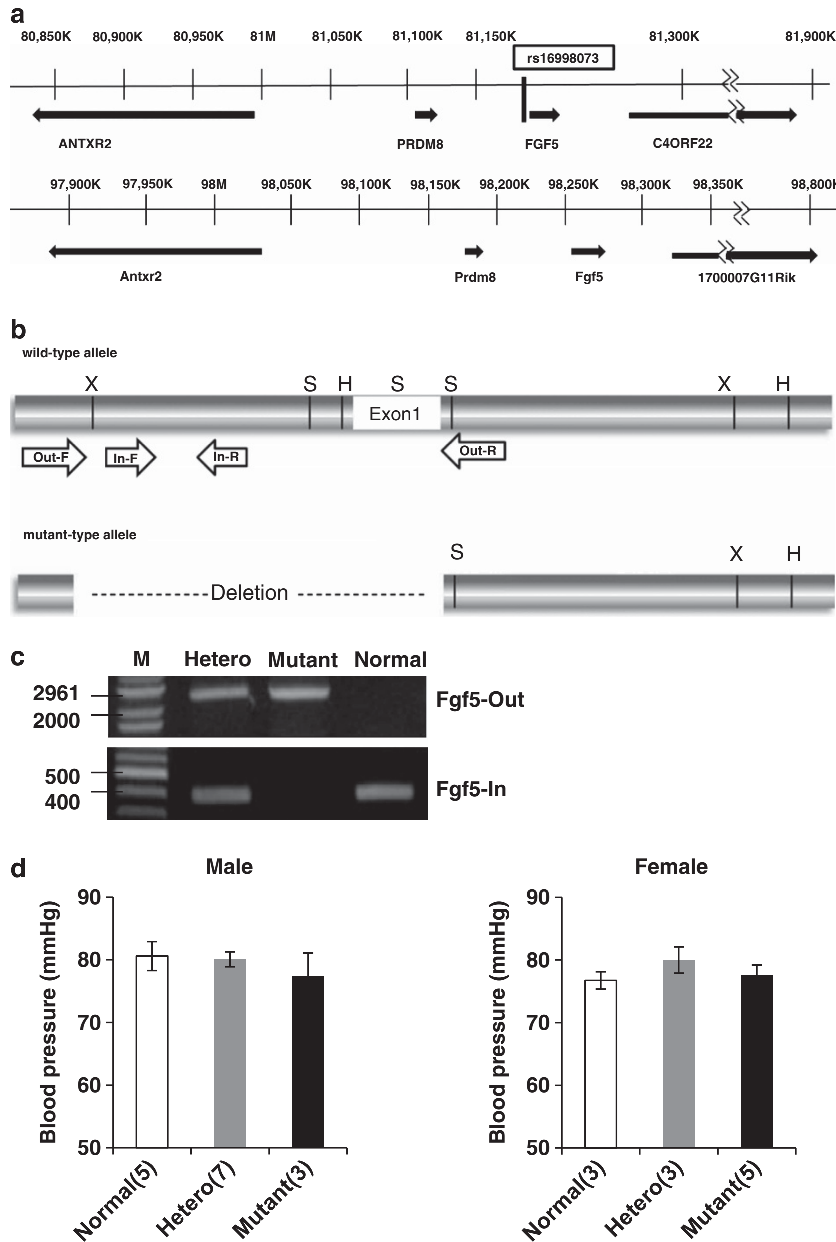

Figure 1 Candidate genes near a lead single-nucleotide polymorphism (rs16998073) in the human 4q21 locus associated with blood pressure. (**a**) Candidate genes in human 4q21 (upper panel) and orthologous genes in mouse 5qE3 locus (lower panel) equivalent to human 4q21. Mouse *1700007G11Rik* matches to human chromosome 4 open reading frame 22. (**b**) Structure of fibroblast growth factor 5 (*FGF5*) gene in wild-type allele and mutant type allele (X, *Xho*I; S, *Sma*I; H, *Hin*dIII). *Fgf5* primers (In-F, forward In primer; In-R, reverse In primer; Out-F, forward Out primer; Out-R, reverse Out primer) used for PCR genotyping were presented on the gene structure. (**c**) Gel electrophoresis for *Fgf5* genotyping of progenies from B6.C-*Fgf5*^{go}/J breeding (M, molecular weight marker; Hetero, heterozygote; Mutant, mutant homozygote). (**d**) Blood pressures of male and female B6.C-*Fgf5*^{go}/J mice measured in the carotid artery for 40 min (from 40 min to 80 min after anesthesia). The number in the parenthesis indicates the number of mice used for the assay. The values measured for the blood pressure are given as the mean ± s.e.m., and the statistics were analyzed by the Kruskal–Wallis test.

FGF5 was considered the strongest candidate of the four genes.[5,7,8] To determine whether *FGF5* is a causative gene in 4q21, we examined B6.C-*Fgf5*^{go}/J mice, a null mutant of the *Fgf5* knockout in which exon 1 of *Fgf5* is deleted.[12] Progeny were generated by breeding heterozygotes and genotyped using two primer sets to distinguish the mutant and wild-type alleles (Figures 1b and c and Table 1).

The blood pressures of *Fgf5* mice of each genotypes were measured from the carotid artery in both genders. As shown in Figure 1d, there was no differences in blood pressure between genotypes of *Fgf5*-knockout mice (females $P=0.517$, males $P=0.660$).

Selection of siRNA for *Antxr2* silencing

Because blood pressure is unchanged in *Fgf5*-knockout mice, we examined *Prdm8* and *Antxr2* by *in vivo* siRNA silencing in mice. Three siRNAs for each gene were synthesized, and their silencing efficacies were analyzed in NIH3T3 cells. *Prdm8* siRNAs, synthesized by Genolution, effected little to no reduction in NIH3T3 cells

Table 1 Genotyping primers for *Fgf5*-knockout mice and quantitative real-time PCR primers for *Antxr2* and *Prdm8*

	Forward sequence	*Reverse sequence*
Genotyping		
Fgf5-Out	5′-AAATGGTGGGAGCCACATAA-3′	5′-GTGGGTGGCTAGTCTTTTCG-3′
Fgf5-In	5′-ATAACAGCCCCAGCATTACG-3′	5′-TCAGGCTGCCCTCTAAGAAA-3′
Real-time PCR		
Antxr2	5′-CCGTTAAGCCAGTTGGAGAA-3′	5′-CCGTCCGTCAAAGCAATTAT-3′
Prdm8	5′-AAACACGTGCACACACAGGT-3′	5′-CAATCCAAAATCCACCATCC-3′
Gapdh	5′-GCATGGCCTTCCGTGTTC-3′	5′-ATGTCATCATACTTGGCAGGT-3′

Abbrevitions: ANTXR2, anthrax toxin receptor 2; FGF5, fibroblast growth factor 5; Gapdh, Glyceraldehyde 3-phosphate dehydrogenase; PRDM8, PR domain-containing 8.

Table 2 siRNAs used for silencing target genes and their silencing efficacies in NIH3T3 cells

siRNA	*Sequence*	*Efficacy (%)*
Antxr2 siRNA-1 (Genolution)	**5′ ACUUGAAGGACUCAAUUCCUU 3′**	**64.6**
*Antxr2*siRNA-2 (Genolution)	5′ UUUCAAUCCAGUUAUUUGCUU 3′	52.3
*Antxr2*siRNA-3 (Genolution)	5′ UUGUAUAUGUGCUGUUUGCUU 3′	46.7
Prdm8 siRNA-1 (Genolution)	5′ AAGAUGUAAGGUACUGUUCUU 3′	3.4
Prdm8 siRNA-2 (Genolution)	5′ UAUAUAAGCUUCGAGAUUCUU 3′	No reduction
Prdm8 siRNA-3 (Genolution)	5′ AAUUCAGUCAGUUCUUUCCUU 3′	No reduction
Prdm8 siRNA-1 (Santa Cruz)	5′ UUCUGAGGAACCAUUUGCCtt 3′	
Prdm8 siRNA-2 (Santa Cruz)	5′ AAUUCAGUCAGUUCUUUCCtt 3′	18[a]
Prdm8 siRNA-3 (Santa Cruz)	5′ UCAUGAUACAUGCACUUCCtt 3′	
Negative control	5′ UUCUCCGAACGUGUCACGUUU 3′	

Abbrevitions: ANTXR2, anthrax toxin receptor 2; PRDM8, PR domain-containing 8; siRNA, small interfering RNA.
Bold indicates siRNA used for *in vivo* silencing.
[a]Santa Cruz provides the mixture of three siRNAs so that the silencing efficacy shown here indicates the effect of the mixure of three *Prdm8* siRNAs.

(Table 2). Additional *Prdm8* siRNAs were purchased from Santa Cruz Biotechnology but induced insufficient reduction of *Prdm8* mRNA (18.3%) in NIH3T3 cells. Thus, *Prdm8* was not examined further by *in vivo* siRNA silencing in mice.

In contrast, the three *Antxr2* siRNAs reduced mRNA levels by 64.6, 52.3 and 46.7% in NIH3T3 cells, respectively (Table 2). The most efficient siRNA was synthesized on a large scale for use in the *in vivo* experiments.

Decrease in *Antxr2* mRNA by siRNA silencing in mice

Before siRNA was injected into the mice, *Antxr2* mRNA levels in the heart, liver, kidney and lungs of normal mice were measured by reverse-transcription PCR. *Antxr2* was expressed in most tissues (Figure 2a).

Antxr2 siRNA that was precoated with polyethylenimine was injected into tail veins, and *Antxr2* mRNA levels were measured 24 h after injection. The siRNA injection was injected 1, 2 or 3 times, once per day. The effects of *Antxr2* siRNA in the various tissues are shown in Figure 2b. *Antxr2* mRNA levels in twice-injected mice decreased significantly in the heart by 21.6% (0.78-fold ± 0.07, $P=0.016$).

Next, Antxr2 protein levels were analyzed in siRNA-injected mice. Kidney and lung tissues in normal mice expressed abundant amounts of Antxr2 (Figure 2c). Thus, changes in protein levels in kidney and lung tissues were analyzed in siRNA-injected mice by western blot. Antxr2 decreased significantly in kidney (case $n=5$, control $n=6$, $P=0.015$) but not in lung tissue (case $n=8$, control $n=8$, $P=0.798$, data not shown) (Figure 2d).

Increase in blood pressure in *Antxr2* siRNA-injected mice

Blood pressure was measured in mice that were injected with control or *Antxr2* siRNA (Figure 3). Blood pressure changed significantly in singly injected mice (control $n=15$, case $n=16$, $P=0.024$). Moreover, double injection of *Antxr2* siRNA increased-blood pressure even more than single injection (control $n=17$, case $n=19$, $P<0.001$)—82.74 mm Hg in *Antxr2* siRNA-injected mice versus 74.44 mm Hg after control siRNA treatment. DBP was 69.71 mm Hg in the controls and 77.95 mm Hg in the cases and the SBP was 78.59 mm Hg in the controls and 86.68 mm Hg in the cases by double injection. However, triple injection over 3 days did not alter blood pressure (control $n=6$, case $n=6$, $P=0.699$).

DISCUSSION

To identify a causative gene in the 4q21 locus, changes in blood pressure were examined in mice in which gene expression was altered by knock out or siRNA silencing. Of the candidate genes, silencing *Antxr2* modulated blood pressure change, indicating that *ANTXR2* is the causative gene in the 4q21 GWAS locus that regulates individual differences in blood pressure in humans.

ANTXR2 is a type 1 transmembrane protein that binds laminin and collagen IV in the basement membrane via a von Willebrand factor type A domain.[10] The rare human disease systemic hyalinosis is caused by mutations in *ANTXR2*.[16] Systemic hyalinosis is an autosomal-recessive disease that encompasses two syndromes—infantile systemic hyalinosis and juvenile hyaline fibromatosis—and is characterized by multiple subcutaneous skin nodules, gingival hypertrophy, joint contractures and hyaline deposition.[16] Infantile systemic hyalinosis and juvenile hyaline fibromatosis have been implicated in the perturbation of basement-membrane assembly as causes of the deposition of perivascular hyaline.[16] The hyaline material that is deposited between the endothelial cells and pericytes in infantile systemic hyalinosis and juvenile hyaline fibromatosis might result from the leakage of plasma components through the basement membrane into the perivascular space.

Furthermore, in the uterus and cervix of the *Antxr2*-knockout mice, there is aberrant deposition of extracellular matrix proteins, such as type I collagen, type VI collagen and fibronectin.[17] These data implicate *ANTXR2* in extracellular matrix assembly, including the basement membrane of vascular endothelium, which is essential for angiogenesis.

In addition, *ANTXR2* expression in endothelial cells is upregulated during human-capillary morphogenesis in three-dimensional-collagen matrices; thus, *ANTXR2* was initially termed capillary morphogenesis gene 2.[10] Moreover, endothelial cells depend on proper *Antxr2* expression for proliferation and capillary network formation.[18] Decreased *ANTXR2* expression in human umbilical vein endothelial cells inhibits proliferation and limits their capacity to form capillary-like networks, whereas overexpression of *ANTXR2* increases proliferation and capillary-like networks, implicating *ANTXR2* in angiogenesis.

The reduction in the number or density of microvessels, known as microvascular rarefaction, is frequent in essential hypertension and in the spontaneously hypertensive rat.[19–21] Because microvascular rarefaction precedes elevations in blood pressure in some cases, it has been assumed to cause such increases.

Thus, we propose that *ANTXR2* influences blood pressure through angiogenesis in relation to basement-membrane assembly. We

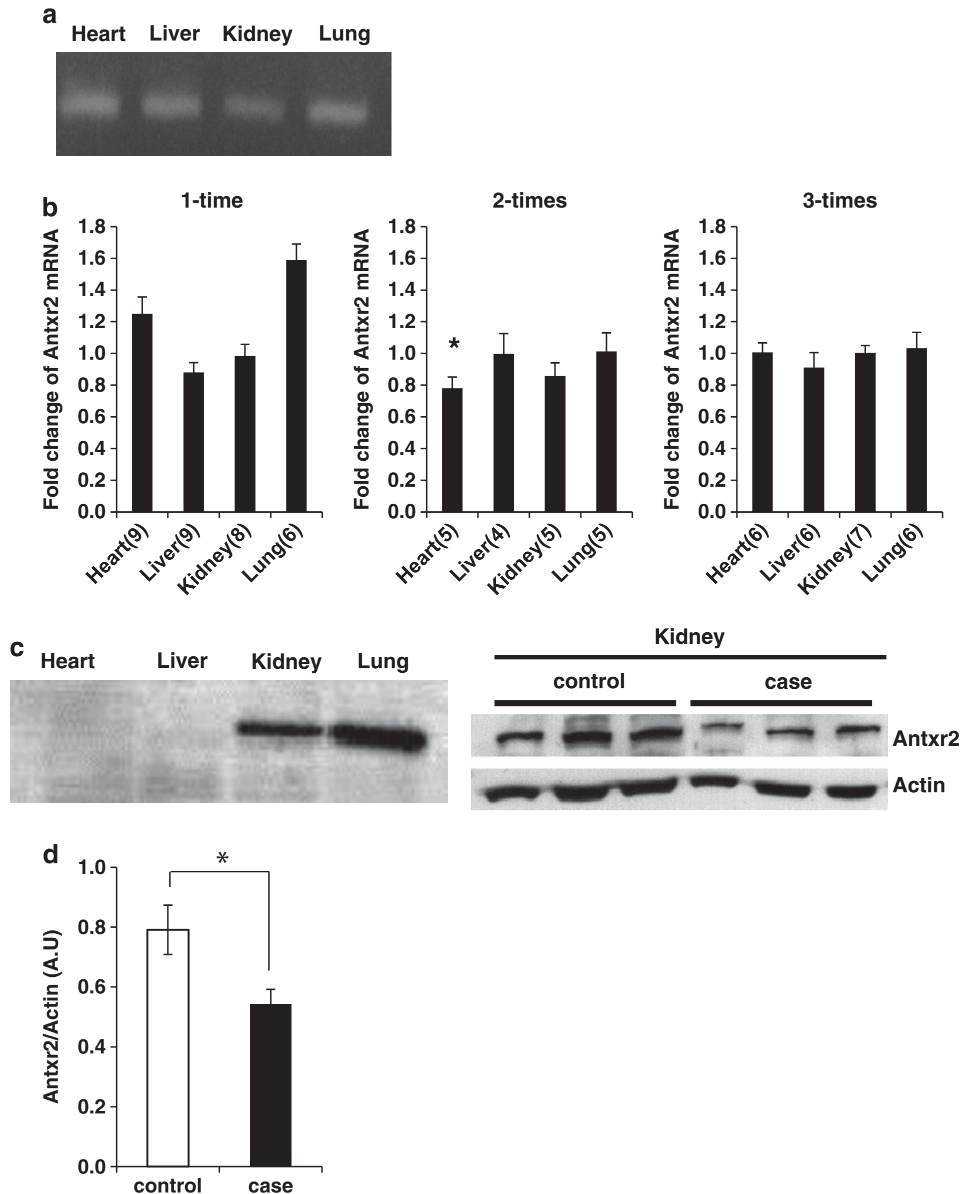

Figure 2 Decrease in anthrax toxin receptor 2 (*ANTXR2*) mRNA and protein by small interfering RNA (siRNA) delivery in mice. (**a**) Expression of *Antxr2* mRNA in normal mouse tissues. (**b**) Fold changes of the *Antxr2* mRNA level in the tissues of mice injected with siRNA once, twice and three times. The number in the parentheses indicates the number of mice used for the assay. The star on the bar graph of the heart indicates the statistical significance ($P<0.05$) for the difference between case and control. (**c**) Western blot for *Antxr2* protein in a normal mouse (left panel) and a mouse injected twice with siRNA (right panel). (**d**) Decrease of Antxr2 protein in kidney of mice injected twice with *Antxr2* siRNA. The star on the bar graph of the heart indicates the statistical significance ($P<0.05$) for the difference between case and control.

speculate that the lower *Antxr2* expression by siRNA silencing in this study resulted in decreases in the proliferation of endothelial cells and altered the basement membrane, preventing the appropriate capillary network from forming and leading to microvascular rarefaction and increased-blood pressure.

Alternatively, we hypothesize that *ANTXR2* regulates blood pressure through signaling by matrix metallopeptidase 14 (membrane-inserted) (MMP14)-matrix metallopeptidase 2 (MMP2)-endothelin-1 in the extracellular matrix.[18,19] *ANTXR2* is known as a positive regulator of MMP14 that activates MMP2.[17] Recently, it was reported that vascular MMP2 mediated the vasoconstricting effects of endothelin-1 by activating it.[22] Consequently, the increased expression of *ANTXR2* results in a dose-dependent increase in active-MMP levels. In this context, *Antxr2* siRNA-injected mice should have developed dilated-blood vessels, leading to the decrease in blood pressure. However, *Antxr2* siRNA-injected mice experienced increased-blood pressure. The mechanism of this phenomenon is unknown, thus meriting further examination of the function of *Antxr2* in blood vessels with regard to MMP14-MMP2-endothelin-1 signaling.

GWASs have discovered genetic loci that confer risk in many diseases. However, most lead single-nucleotide polymorphisms in these GWASs reside between protein-coding genes.[23] In addition, multiple genes lie near to the associated signal in many cases. Thus,

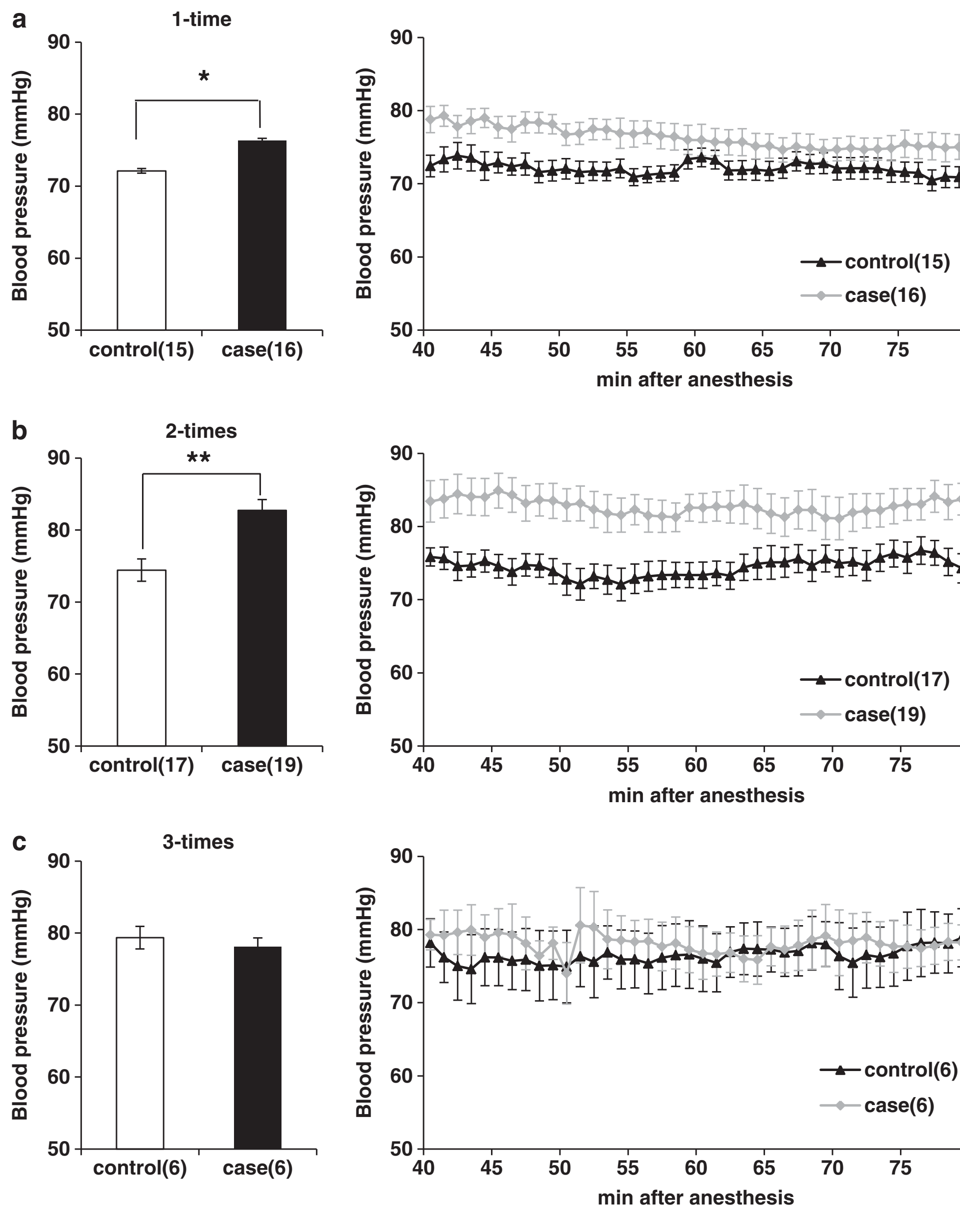

Figure 3 Blood pressure of anthrax toxin receptor 2 small interfering RNA (siRNA)-injected mice. (**a**) Measurement of blood pressure during the interval of 40–80 min after anesthesia in mice injected once with siRNA (control = 15, case = 16, $^{*}P = 0.024$). (**b**) Measurement of blood pressure in mice injected twice with siRNA (control = 17, case = 19, $^{**}P < 0.01$). (**c**) Measurement of blood pressure in mice injected three times with siRNA (control = 6, case = 6, $P = 0.699$). Bar graph shows the mean and variance (mean ± s.e.m.) in blood pressures in mice injected once, twice and three times.

identifying causative genes in risk-related genomic regions has been challenging. To identify target genes in GWAS loci, in genetically manipulated mice, techniques such as conventional knockouts and knockouts, have been used. However, the construction of such mice is expensive and time consuming.

In contrast, *in vivo* siRNA silencing is less expensive and much faster, rendering it suitable for the functional validation of GWAS results. Despite these advantages, siRNA-based methods have several limitations. As shown in this study, *Prdm8* function could not be examined by *in vivo* siRNA silencing because we could not develop *Prdm8* siRNAs that silenced it in cells. In such cases, the over-expression of a target gene by an adeno-associated virus can be used to identify the causative gene in a GWAS locus.[24]

In summary, four candidate genes in the 4q21 locus were examined to identify a causative gene, of which the silencing of *Antxr2* caused blood pressure changes in siRNA-injected mice, suggesting that *ANTXR2* is a causative gene in the 4q21 GWAS-blood pressure locus. We hypothesize that *ANTXR2* regulates blood pressure through angiogenesis or vascular-smooth muscle contraction. Further functional study of *ANTXR2* might identify a new pathway in the regulation of blood pressure, thereby increasing our understanding of the etiology of essential hypertension.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Hypertension Research website (http://www.nature.com/hr)