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Original

Role of Gremlins in the Aortic Arch of Spontaneously Hypertensive and Hyperlipidemic Rats

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Abstract: Atherosclerosis is a lifestyle-related disease that plays a major role in cardiovascular disease. Recently, we found that gene expression of *Gremlin 2*, an antagonist of bone morphogenetic protein (BMP), was significantly increased in the aorta of spontaneously hypertensive and hyperlipidemic rats (SHHRs) fed a high-fat, 30% sucrose solution diet (HFDS). However, the role of *Gremlin 1* (Grem1) and *Gremlin 2* (Grem2) in the aortic arch of rats under hypertensive, hyperlipidemic, and hyperglycemic conditions remains unclear. Therefore, in the present study we investigated the molecular role of Gremlins in the aorta of SHHRs. Four-month-old male Sprague-Dawley rats and SHHRs were fed a normal diet or the HFDS *ad libitum* for 4 months. Then, gene and protein expression was analyzed using quantitative polymerase chain reaction and western blotting, respectively. Grem1 and Grem2 protein expression was increased, whereas phosphorylated Smad1/5 protein expression was low, in the aorta of SHHRs fed the HFDS. In addition, the expression of the downstream gene targets of BMP, namely inhibitor of DNA binding 1 (*Id1*) and atonal homolog 8 (*Atoh8*), was decreased in aortas of SHHRs fed the HFDS. Furthermore, mRNA expression of *Snail,* α-smooth muscle actin (*αSMA*), and *Fibronectin* was increased in SHHRs fed the HFDS. These findings suggest that upregulation of Gremlins attenuates the activation of BMP signaling, which contributes to fibrogenesis of the aorta.

Key words: atherosclerosis, Gremlin 1, Gremlin 2, bone morphogenetic protein, spontaneously hypertensive and hyperlipidemic rat (SHHR)

Introduction

Atherosclerosis is a lifestyle-related disease characterized by intimal plaque, which leads to coronary heart disease and cerebrovascular disease[1,2]. The risk factors for atherosclerosis include hypertension, hyperlipidemia, and diabetes, and, with the increase in the prevalence of these diseases, the number of atherosclerosis patients has also increased. Atherosclerosis progresses

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silently until the occurrence of vascular stenosis or occlusion and cardiovascular abnormalities[3]. Therefore, the establishment of diagnostic procedures and treatment for atherosclerosis is a significant issue. It is necessary to identify a novel biomarker for the detection of atherosclerosis in its early stages. However, the molecular mechanism (s) underlying the early stage of development of atherosclerosis remains unclear. Therefore, elucidation of the mechanism involved in the early stages of atherosclerosis is important for the identification of new biomarkers.

Previously, we established a spontaneously hypertensive, hyperlipidemic rat (SHHR) strain by cross-breeding Sprague-Dawley (SD)-derived hyperlipidemic rats with Wistar-Kyoto-derived spontaneously hypertensive rats (SHR)[4]. In SHHRs fed a normal diet, systolic blood pressure is $>$ 150 mmHg and total cholesterol levels are $>$ 150 mg / dl. SHHRs fed a high-fat, 30% sucrose solution diet (HFDS) develop lipid deposits in the aortic arch. This model is widely used for the study of vascular disorders and arteriosclerosis[4–8].

Recently, we reported that the gene expression of *Gremlin 2* (*Grem2*) was increased significantly in the aorta of SHHRs fed the HFDS[9]. Gremlins are members of the bone morphogenetic protein (BMP) antagonist family[10, 11]. *Gremlin 1* (*Grem1*) was isolated using a *Xenopus* expression-cloning screen[10] and is expressed in the skeletal muscle, lung, kidney, and pancreas. For example, *Grem1* has been shown to be localized in the endothelial cells of the pulmonary artery and in tubular cells of the kidney[12–14]. *Grem1*$^{-/-}$ mice exhibit germline lethality, whereas in *Grem1*$^{+/-}$ mice the bone is not fully developed, resulting in severe developmental defects compared with wild-type mice[15]. In addition, *Grem1* is highly expressed in idiopathic pulmonary fibrosis (IPF), diabetic nephropathy, and fibrotic processes in the liver and heart[16–19]. In these patients, BMP signaling activity is attenuated, or transforming growth factor (TGF) β signaling activity is concomitantly enhanced. Each of these diseases is known to involve further fibrosis due to TGFβ signaling[20–23]. Thus, it is suggested that *Grem1* is associated with tissue fibrogenesis in patients with IPF, diabetic nephropathy, and fibrosis of the heart and liver. Conversely, *Grem2* was discovered in mouse embryonic stem cells by gene trapping[24], and has been observed in lung mesenchyme[25]. *In vivo* studies have implicated *Grem2* in follicle development, placode neurogenesis, osteogenic differentiation, and craniofacial patterning[11, 26-28]. There are no reports of studies performed in mice with genetically modified *Grem2*.

We hypothesized that *Grem1* and *Grem2* could promote fibrogenesis of the aortic arch of SHHRs and contribute to the occurrence of atherosclerosis when the rats are fed HFDS. To examine this, in the present study we measured *Grem1* and *Grem2* expression, as well as BMP signaling activity, in the aorta of SD rats and SHHRs. In addition, we analyzed the expression of TGFβ target genes to clarify the involvement of *Grem1* and *Grem2* in TGFβ signaling.

Methods

Animals

The present study was performed on 8-month-old male SD rats and SHHRs. Up to 4 months of age, all rats were fed a normal diet (ND) *ad libitum*. Then, rats were divided into four groups of four to six rats each and fed either the ND or HFDS for a further 4 months, as

follows: (i) SD rats fed the ND; (ii) SD rats fed the HFDS (SD-HFDS group); (iii) SHHRs fed the ND; and (iv) SHHRs fed the HFDS (SHHR-HFDS group). Rats in all four groups were allowed *ad libitum* access to the respective diet. The ND consisted of regular rat chow (CE2; CLEA Japan Inc., Tokyo, Japan) comprising 8.9% water, 25.4% protein, 4.4% fat, 4.1% fiber, 6.9% carbohydrate, and 50.3% nitrogen-free extracts, containing 342.2 kcal / 100 g. The HFDS (CLEA Japan Inc.) was comprised of 8.2% water, 23.4% protein, 11.0% fat, 3.8% fiber, 6.3% carbohydrate, and 46.3% nitrogen-free extracts, containing 378.0 kcal / 100 g. Rats were housed in a semi-barrier system under conditions of controlled temperature (23 ± 1°C), humidity (55 ± 5%), and lighting (lights on from 0600 to 1800 hours). At the end of the 4-month experimental feeding period, rats were killed under pentobarbital anesthesia. The entire aortic arch was removed from all animals and used in subsequent experiments. All experiments were conducted according to the Guiding Principles for the Care and Use of Laboratory Animals of Showa University.

Quantitative reverse transcription-polymerase chain reaction analysis

Total RNA was extracted from aortic arch specimens using a commercially available kit (RNeasy Mini Kit; Qiagen, Germantown, MD, USA), following the manufacturer's instructions. The RETROscript Kit (Ambion, Austin, TX, USA) was used to synthesize cDNA from total RNA. The cDNA was amplified using LightCycler SYBR Green Master mix (Roche Diagnostic, Basel, Switzerland) in a LightCycler (Roche Diagnostic). Fluorescence data were analyzed using LightCycler software (Roche Diagnostic). The expression of target genes was normalized against that of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), used as a housekeeping gene, and measured concurrently. The primers used in the present study are listed in Table 1.

Western blot analysis

Aortic arch specimens from each group were homogenized in lysis solution (50 mmol / l Tris-HCl, pH 7.4, 1 mmol / l EGTA, 0.001% leupeptin). The supernatant was collected following centrifugation of samples at 16,100 × *g*, for 30 min at 4°C, and denatured for 5 min at 95–100°C with 0.6% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol. The denatured lysate was resolved by SDS–polyacrylamide gel electrophoresis. The separated proteins were transferred to a hydrophobic polyvinylidene difluoride membrane (GE Healthcare, Uppsala, Sweden) and blocked with 3% skimmed milk in Tris-buffered saline containing Tween 20 (150 mmol / l NaCl, 100 mmol / l Tris-HCl, pH 7.5, 0.5% Tween[20]) for 1 h at room temperature. The membranes were reacted against anti-*Grem1*, anti-*Grem2* (Bioss, Wobun, MA, USA), anti-Smad1 / 5 (Abcam, Tokyo, Japan), anti-phosphorylated (p-) Smad1 / 5 (Cell Signaling Technology, Beverly, MA, USA), and anti-α-tubulin antibodies (Abcam) overnight at 4°C. Specific antigen–antibody complexes were visualized using a horseradish peroxidase-conjugated secondary antibody and ImmunoStar LD (Wako, Osaka, Japan). Densitometric analysis of the blots was performed using a C-DiGit Blot Scanner and Image Studio for the C-DiGit Blot Scanner (Li-Cor, Lincoln, NE, USA).

Table 1. List of primers used for real-time polymerase chain reactions

Gene	Primer (5′–3′)
Grem1	Forward: GTCCACAGCGAAGGACTTGAGGA
	Reverse : CTTCAGCTGCTGGCAGTAGGGTTC
Grem2	Forward: TGTGGATTGTGTCATTCACACAGAGG
	Reverse : CTTTACCAGCACAGCCACCAGGAG
Id1	Forward: TGAACGGCGAGATCAGTGCCTTG
	Reverse : CTGGAGTCCATCTGGTTCCTCAGTG
Atoh8	Forward: GAGATCAAAGCCCTGCAGCAGAC
	Reverse : GTTTGGACAGCTTCTGCCCATAGGA
Snail	Forward: CACCCACACTGGTGAGAAGCCTTT
	Reverse : GGCCTGACACTGGTATCTCTTCACA
αSMA	Forward: CAACCGGGAGAAAATGACCCAGATT
	Reverse : AGTCCAGCACAATACCAGTTGTACG
Fibronectin	Forward: CAATCCTGACACTGGAGTGCTTACC
	Reverse : ATCGGCATGAACCACTTCTTCCAAA
GAPDH	Forward: CTGAGTATGTCGTGGAGTCTA
	Reverse : CTGCTTCACCACCTTCTTGAT

Grem1, Gremlin 1; *Grem2*, Gremlin 2; *Id1*, inhibitor of DNA binding 1; *Atoh8,* atonal homolog 8; *αSMA*, α-smooth muscle actin; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

Statistical analysis

The significance of differences among groups was analyzed using the Tukey–Kramer post hoc test. A 5% level of probability was considered significant.

Results

Grem1 and Grem2 gene and protein expression in the aortic arch

In a previous study, we performed a comprehensive analysis of gene expression in the vascular wall of the aortic arch of SD rats and SHHRs and confirmed that the gene expression of *Grem2* is significantly increased in the vascular wall of SHHRs fed the HFDS[9]. In the present study, to investigate the effects of the HFDS on Gremlin expression levels in the vascular wall of SD rats and SHHRs, gene expression of *Grem1* and *Grem2* was determined using quantitative polymerase chain reaction (PCR) analysis. In addition to increases in *Grem2* expression, we found significant increases in *Grem1* expression in the SHHR-HFDS group (Fig. 1A, B). Furthermore, *Grem1* and *Grem2* proteins were highly expressed in the vascular wall of the SHHR-HFDS group (Fig. 1C). Densitometric analysis revealed significant increases in *Grem1* and *Grem2* protein expression, as determined by western blotting (Fig. 1D, E). These results indicate that *Grem1* and *Grem2* expression is increased in the vascular wall of rats in the SHHR-HFDS

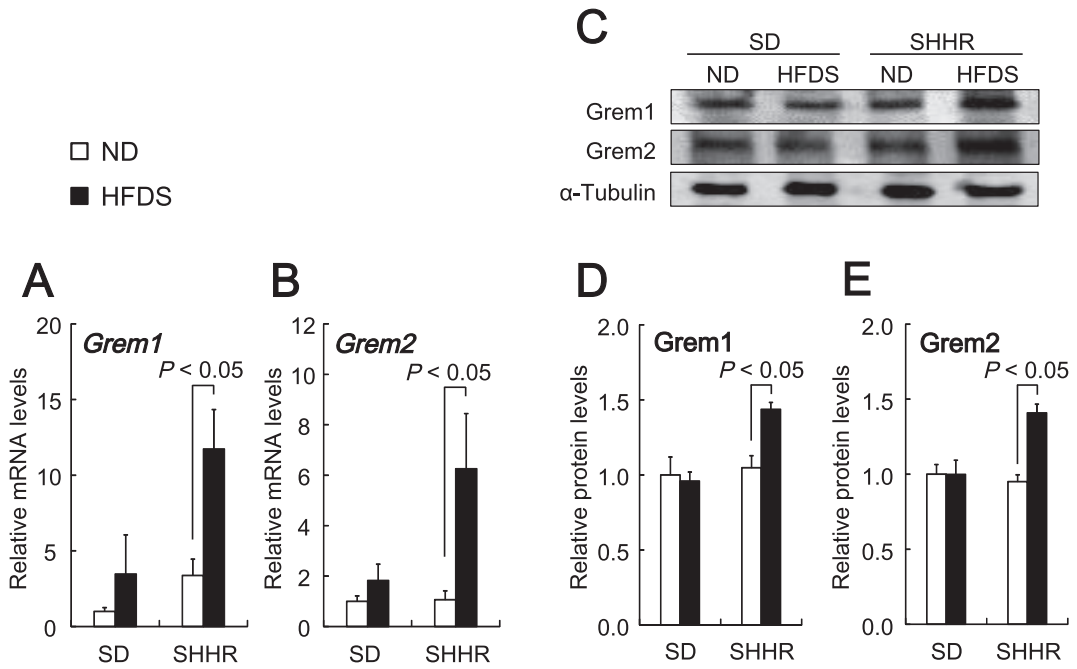

Fig. 1. Gene and protein expression of Gremlin 1 (Grem1) and Gremlin 2 (Grem2) in the aortic arch of Sprague-Dawley (SD) rats and spontaneously hypertensive and hyperlipidemic rats (SHHRs) fed either a normal diet (ND) or a high-fat diet plus 30 % sucrose solution (HFDS) for 4 months. (A) *Grem1* and (B) *Grem2* mRNA expression was measured using quantitative PCR analysis. Data were normalized against glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) expression. (C) Representative images of western blotting for Grem1 and Grem2. α-Tubulin was used as an internal control. (D, E) Densitometric analysis of Grem1 and Grem2 protein expression. Data are the mean ± SE ($n = 4$–6 rats per group). $^{*}P < 0.05$ (Tukey–Kramer post hoc test).

group.

Grem1 and Grem2 expression and Smad1 / 5 phosphorylation in the aortic arch

We next investigated the molecular roles of *Grem1* and *Grem2* in the vascular wall. Under conditions of stimulated BMP signaling, Smad1 / 5 is phosphorylated and forms a complex with Smad4, which regulates the downstream transcription of target genes, such as the inhibitor of DNA binding 1 (*Id1*) and atonal homolog 8 (*Atoh8*)[29]. Therefore, in the present study we analyzed Smad1 / 5 phosphorylation using western blotting. Low levels of p-Smad1 / 5 accumulated in the vascular wall of the SHHR-HFDS group compared with the other three groups, although there was no significant difference in the expression of total Smad1 / 5 proteins (Fig. 2A). Furthermore, levels of p-Smad1 / 5 were significantly lower in the SHHR-HFDS group than in the other groups (Fig. 2B). These findings suggest that the increased expression of *Grem1* and *Grem2* proteins inhibits BMP signaling, resulting in low accumulation of p-Smad1 / 5 in the vascular wall of the SHHR-HFDS group compared with the other three groups.

Downstream gene expression of BMP signaling

Chromatin immunoprecipitation sequencing (ChIP-seq) revealed that Smad1 / 5 proteins bind

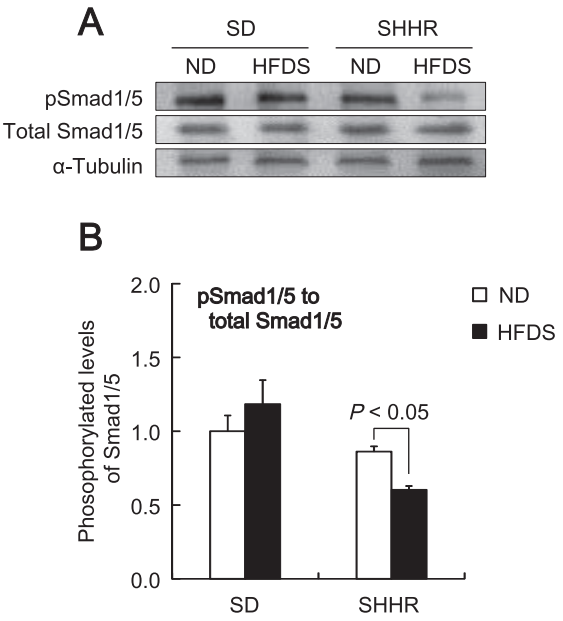

Fig. 2. Protein expression profiles of phosphorylated (p-) Smad1/5 and total Smad1/5 in the aortic arch of Sprague-Dawley (SD) rats and spontaneously hypertensive and hyperlipidemic rats (SHHRs) fed either a normal diet (ND) or a high-fat diet plus 30 % sucrose solution (HFDS) for 4 months. (A) Representative western blotting images. α-Tubulin was used as an internal control. (B) Expression ratios for p-Smad1/5 to total Smad1/5, as determined by densitometric analysis, in SD rats and SHHRs. $^{*}P < 0.05$ (Tukey–Kramer post hoc test).

to the promoter regions of *Id1* and *Atoh8* with BMP9 stimulation in human umbilical vein endothelial cells[30]. To clarify the effects of *Grem1* and *Grem2* on downstream gene expression in BMP signaling, quantitative PCR analysis was used to evaluate *Id1* and *Atoh8* expression. As expected, low levels of *Id1* and *Atoh8* expression were seen in the vascular wall of the SHHR-HFDS group (Fig. 3A, B). These results indicate that a low level of p-Smad1/5 does not regulate the transcription of *Id1* and *Atoh8*, and results in decreased transcription in the SHHR-HFDS group.

TGFβ target gene expression in the vascular wall

The association of *Grem1* and *Grem2* with BMP signaling led us to hypothesize that these Gremlins may enhance the activity of TGFβ signaling in the vascular wall of SD rats and SHHRs. It has been reported that *Grem1* is highly expressed and TGFβ signaling is activated in the lungs of IPF patients[17]. To investigate the activity of the TGFβ signaling pathway in the two rat strains in the present study, expression of the TGFβ target genes Snail, α-smooth muscle actin (αSMA), and Fibronectin was determined. The expression of all three genes was increased significantly in the vascular wall of the SHHR-HFDS group compared with the other three groups (Fig. 4). These data indicate that the TGFβ signaling pathway is activated in the aortic arch of the SHHR-HFDS group.

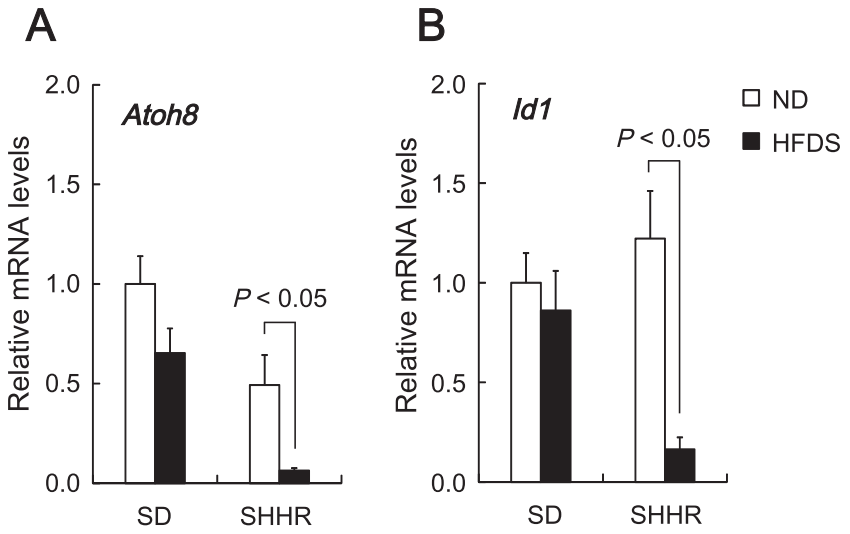

Fig. 3. Downstream transcription of bone morphogenetic protein (BMP) signaling in the aortic arch of Sprague-Dawley (SD) rats and spontaneously hypertensive and hyperlipidemic rats (SHHRs) fed either a normal diet (ND) or a high-fat diet plus 30% sucrose solution (HFDS) for 4 months. (A) Inhibitor of DNA binding 1 (*Id1*) and (B) atonal homolog 8 (*Atoh8*) mRNA expression was determined using quantitative PCR analysis. Data were normalized against glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) expression. Data are the mean ± SE ($n = 4$–6 rats per group). $^{*}P < 0.05$ (Tukey–Kramer post hoc test).

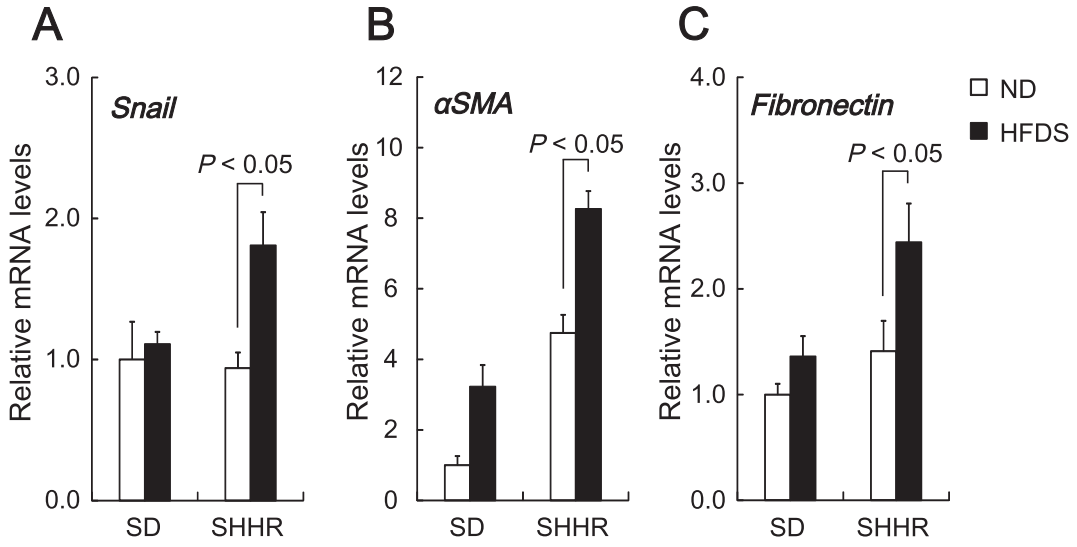

Fig. 4. Expression of transforming growth factor (TGF) β target genes in the aortic arch of Sprague-Dawley (SD) rats and spontaneously hypertensive and hyperlipidemic rats (SHHRs) fed either a normal diet (ND) or a high-fat diet plus 30% sucrose solution (HFDS) for 4 months. (A) Snail, (B) α-smooth muscle actin (αSMA), and (C) Fibronectin mRNA expression was determined using quantitative PCR analysis. Data were normalized against glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) expression. Data are the mean ± SE ($n = 4$–6 rats per group). $^{*}P < 0.05$ (Tukey–Kramer post hoc test).

Discussion

Gremlins are members of the BMP antagonist family[10]. They bind to BMP ligands, such as BMP2 and BMP4, preventing the interaction between the BMPs and their receptors[10, 11]. When stimulated by BMP signaling, Smad1 and Smad5 are phosphorylated and form complexes[29]. These complexes are recruited to the promoters of target genes, regulating the expression of genes such as *Id1* and *Atoh8*[30]. Therefore, Gremlins likely suppress the activity of Smad1 / 5 signaling by inhibiting BMP signaling in various cells. In addition, it is likely that the Smad1 / 5 signaling suppressed by Gremlins is coupled to the TGFβ signaling cascade[31, 32]. In the present study, there was a significant increase in *Grem1* and *Grem2* gene and protein expression in the SHHR-HFDS group compared with the other groups. Immunostaining of the aortic arch of the SHHR-HFDS group revealed that *Grem1* and *Grem2* were predominantly localized in vascular smooth muscle cells (data not shown). In the SHHR-HFDS group, low levels of p-Smad1 / 5 proteins were observed, and there was a marked decreased in *Id1* and *Atoh8* expression. These findings suggest that the high expression of *Grem1* and *Grem2* attenuated the phosphorylation of Smad1 / 5, which failed to regulate the expression of *Id1* and *Atoh8* in the aortic arch in the SHHR-HFDS group.

We next focused on how downregulation of BMP signaling affects the aortic arch in the SHHR-HFDS group. Gremlins have been shown to be involved not only in embryonic development, but also in fibrogenesis in the lung, heart, kidney, and liver[16–19]. For example, *Grem1* is highly expressed in the lungs of patients with IPF, in which TGFβ secretion and αSMA expression are higher than in healthy adult lung fibroblasts[17]. In the present study, expression of Snail, *αSMA*, and *Fibronectin*, all of which are TGFβ target genes, was significantly increased in the aortic arch of rats in the SHHR-HFDS group. Because the expression of these target genes increases with the progression of fibrosis[33–35], fibrogenesis would also occur in the aortic arch of rats in the SHHR-HFDS group. Interestingly, these target genes are involved in the endothelial–mesenchymal transition (EMT)[36–38]. Recent studies have demonstrated that activated fibroblasts can arise from endothelial cells during fibrosis via the EMT and have significant roles in cardiac and renal fibrosis[39, 40]. Therefore, the fibrogenesis in the aortic arch of rats in the SHHR-HFDS group in the present study would be attributable to atherosclerosis in the EMT. Notably, the downregulation of BMP signaling and upregulation of TGFβ target gene expression were not observed in the SD-HFDS group. These results suggest that hypertensive, hyperlipidemic, and hyperglycemic factors play a key role in the fibrogenesis of the aortic arch.

The process of atherosclerosis is triggered by an increase in plasma cholesterol levels, especially low-density lipoprotein cholesterol (LDL-C). Oxidized LDL-C modified by reactive oxygen species has a chemotactic effect on monocytes and induces the expression of monocyte chemoattractant protein-1 (MCP-1), granulocyte–macrophage colony-stimulating factor, and adhesion molecules[41–43]. Thus, oxidized LDL-C stimulates the recruitment of monocytes to the lesion site, as well as the proliferation of macrophages, both of which contribute to progression of the inflammatory response[44–46]. Importantly, LDL-C is not found in the blood of normal rats, but

is seen in the blood of SHHRs[4]. A previous study found that lipid deposition and production of MCP-1 and CD68, a macrophage marker, were observed only in the intimal surface in the SHHR-HFDS group[9]. Because MCP-1 is involved in the initiation and proliferation of monocyte recruitment in the vascular intima[47], the aortic arch of rats in the SHHR-HFDS group would represent the early stage in the development of atherosclerosis. The results of the present study suggest a role for *Grem1* and *Grem2* in the fibrogenesis of the vascular wall in SHHRs. The results also indicate a possible molecular link between *Grem1* and *Grem2* and BMP signaling. These findings may contribute to the development of novel biomarkers to detect atherosclerosis in the early stages.

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Conflict of interest disclosure

The authors declare they have no conflicts of interest to report.

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