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Cytoglobin overexpression facilitates proliferation and migration of vascular smooth muscle cells

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Abstract: Cytoglobin, a recently discovered globin, is expressed in vascular smooth muscle cells (VSMCs). Loss of cytoglobin provides a protective effect on vascular reconstruction but the effect of its overexpression is unclear. The aim of the study was to investigate the effect of cytoglobin overexpression on the migration and proliferation of VSMCs and possible mechanisms. We detected the expression of cytoglobin in hypertensive and normotensive rat aortas, with negative feedback regulation between cytoglobin and hypertension observed. The expression of cytoglobin was significantly decreased in hypertensive rats compared to normotensive rats, but VSMCs overexpressing cytoglobin displayed increased cell migration and proliferation, which led to a phenotypic switch. The increased expression of matrix metalloproteinase 9 and collagen Ia suggests a role for cytoglobin in extracellular matrix remodeling. Increased expression of proliferating cell nuclear antigen and decreased expression of p27 implies that cytoglobin is involved in modulating VSMC proliferation. Our findings indicate that cytoglobin may play an important role in vascular wall remodeling.

Keywords: cytoglobin; vascular smooth muscle cells; hypertension; cell migration; cell proliferation

Abbreviations: vascular smooth muscle cells (VSMCs); extracellular matrix (ECM); cytoglobin (CYGB): matrix metalloproteinase 9 (MMP9); proliferating cell nuclear antigen (PCNA); smoothelin (SMTN)

INTRODUCTION

Hypertension is the primary risk factor for cardiovascular diseases such as stroke, heart attack, heart failure, aneurysm, myocardial infarction and vascular dementia [1,2]. The pathological changes resulting from hypertension include: hyperplasia, migration, apoptosis of VSMCs and deposition of the ECM in vessels [3]. VSMCs, which constitute the bulk of the vascular wall, are critically involved in these processes due to their highly plastic and dynamic features and their ability to undergo phenotypic differentiation [4-6]. However, under disease conditions such as atherosclerosis and hypertension, the phenotype of VSMCs shifts from contractile to synthetic, with an increased level of migration, proliferation and decreased contractile marker expression. Excessive proliferation of VSMCs and deposition of the ECM contribute to thickening of vessel walls and enhanced vascular stiffness [7,8]. At the molecular and cellular levels, vascular hyperreactivity, remodeling, and stiffening involve changes in cytoskeletal organization, cell-to-cell connections, cell growth, calcification, inflammation and rearrangement of VSMCs [9,10].

CYGB, a new member of the globin family, was discovered in 2001 as a protein associated with stellate cell activation [11]. Accumulating evidence has shown that CYGB is not only expressed in the liver, but also in many other tissues including breast, pancreas, lungs and blood vessels [12-14]. In blood vessels, it is mainly expressed in VSMCs [15].

Despite numerous studies, CYGB function remains poorly understood. In the kidney, recent studies indicate cytoprotective functions of CYGB, potentially associated with direct or indirect antioxidant properties [16]. CYGB might also contribute to cell-mediated nitric oxide (NO) metabolism through NO deoxygenation to nitrate in the presence of molecular oxygen or nitrite reduction to NO under hypoxia [17-19]; deletion of CYGB in VSMCs causes an increase in NO. It has also been reported that CYGB is expressed in blood vessels primarily in differentiated medial VSMCs, where it regulates neointima formation and inhibits apoptosis after injury. This is supported by data from CYGB knockout mice that displayed little evidence of neointimal hyperplasia in contrast to their wild-type littermates four weeks after complete ligation of the left common carotid [15].

In this study, we evaluated the expression of the CYGB gene in the aorta between hypertensive and normotensive rats. We further investigated the effect of *CYGB* overexpression on the migration and proliferation of VSMCs, as well as on the expression of extracellular matrix components, which may help to provide deeper insight in the pathogenesis of hypertension.

MATERIALS AND METHODS

Ethics Statement

Animal care and experimental protocols were in accordance with the Animal Management Rules of China (55, 2001, Ministry of Health, China). All animal experimental procedures were performed strictly according to protocols approved by the Committee for Animal Care and Use (Ethics code SCXK Shanghai 2018-0004).

Rat model of hypertension

Sprague-Dawley (SD) male rats, 18 weeks old, weighing 240±20 g were purchased from the Shanghai Laboratory Animal Research Center (Shanghai, China). The rats were housed at 25°C±1°C and humidity 65%-70%, and fed with standard laboratory food and water. SD rats (n=6) were randomly divided into control and hypertension groups. Animals were anesthetized by isoflurane inhalation. Hypertension was induced by bilateral renal artery stenosis using a miniature sliver clip (0.25 mm). Animals from the control group were sham operated. Blood pressure was measured by the tail-cuff method with a BP-2010 Series Blood Pressure Meter (Softron Biotechnology, Beijing, China). Systolic blood pressure above 140 mm Hg was considered high blood pressure. Aortas were harvested from rats 4 weeks post surgery.

VSMC culture

Primary VSMCs were cultured as previously described [20]. The purity of VSMCs was confirmed by immunofluorescent detection of alpha smooth muscle actin expression (Beyotime Biotechnology, China). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% pen/strep (Thermo Fisher Scientific, USA) at 37°C and 5% CO_2 in a humidified incubator (Thermo Fisher Scientific, USA).

Lentiviral infection

To generate the *CYGB* expression construct (*CYGB*-OE), the *CYGB* open reading frame was cloned into a pLVX-IRES-ZsGreen1 lentiviral vector (Genepharma, China). An empty vector was used as a negative control (NC). HEK293T cells were co-transfected with three helper plasmids (PMDL, VSVG, REV) and with either the expression construct *CYGB*-OE or the empty vector, using LentiFit (HANBIO, China) according to the manufacturer's instructions (Asia-vector Biotechnology, China). VSMCs were cultured in HEK293T supernatants collected 72 h after transfection, which contained *CYGB* overexpressing lentivirus or contol lentivirus. Stable cell lines were selected with puromycin (8 ng/mL), and Western blotting was used to confirm CYGB protein expression.

Real time PCR

Aorta samples were lysed in TRIzol and cDNA was generated using a PrimeScript RT reagent kit with gDNA eraser (TaKaRa, Japan) according to the manufacturer's instructions. RT-PCR was performed using 2×SGExcel UltraSYBR Mixture with ROX (Sangon Biotech, China) and an Applied Biosystems 7500 Real-time PCR system (Thermo Fisher Scientific, USA). RNA

levels were calculated using the $2^{-\Delta\Delta Ct}$ method. *GAPDH* was used for normalization. The primers used were: *GAPDH* forward primer sequence (5'-3') AGG TCG GTG TAG ACG GAT TTG, reverse primer sequence (5'-3') TAT AGA CCA TGT AGT TGA GGT CA, rat *CYGB* forward primer sequence (5'-3') CTC TCT GGG GTC ATT CTG GA, and reverse primer sequence (5'-3') ATG GCT GTA GAT ACC GC.

Western blot analysis

VSMCs and aorta samples were lysed in RIPA buffer containing a proteinase inhibitor cocktail (Beyotime Biotechnology, China). Lysates were treated with an ultrasonic cell disruption system (SHUNMATECH, China) at 10% intensity 3 times on ice, and centrifuged at 600 g for 5 min at 4°C. The proteins from the supernatants were separated by 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membrane, blocked with 5% nonfat milk in TBST for 1 h and incubated with the corresponding primary antibody at 4°C overnight. The primary antibodies and dilutions were: anti-α-tubulin (1:1000), anti-CYGB (1:2000), anti-MMP9 (1:2000), anti-SMTN (1:2000), anti-calponin (1:2000), anti-PCNA (1:2000), anti-P27 (1:2000), anti-Col Ia (1:2000), anti-Col VIII (1:2000), (all purchased from Proteintech, USA). The membranes were then incubated with corresponding HRP-conjugated goat anti-mouse (1:10000) or goat anti-rabbit secondary antibodies (1:10000) (Proteintech, USA) at room temperature for 1 h. Finally, the proteins were visualized with enhanced chemiluminescence (Absin Bioscience, China) and the signals were quantified with a Tanon image analyzer (Tanon Science, China).

VSMC migration assay

The scratch test was performed as previously reported [21] with some modifications. CYGB OE and NC VSMCs were seeded at a density of 1×10^6 cells/well into a 6-well plate and grown to confluency. The monolayer of VSMCs was scratched using a sterile pipette tip and washed with PBS three times. The VSMCs culture medium was replaced with a serum-free medium. The closure of the wound area was captured with a BioTek citation TM 3 cell Imaging Multi-Mode Reader (BioTek, USA).

Cell cycle analysis

Control and *CYGB*-overexpressing VSMCs were seeded at a density of 5×10^6 cells/dish and incubated for 48 h. Cells were collected, fixed in 70% ethanol for 5 h at 4°C and stained with propidium iodide (50 g/mL) for 30 min at 37°C according to the manufacturer's instructions (Beyotime Biotechnology, China). The cell cycle was analyzed by flow cytometry (BD Biosciences, USA) and the ModFIT LT 5.0 (Verity Software House, USA) was used to determine the cell cycle distribution.

Statistical analysis

The data were expressed as the mean±SD from three independent experiments. Statistical analyses were performed using GraphPad Prism 7. The *P*-value was calculated using Student's *t* test. A $P\leq0.05$ was considered statistically significant.

RESULTS

Decreased *CYGB* expression in hypertension

First, we investigated if hypertension affected both the gene and protein expression of *CYGB*. As shown in Fig. 1A, the expression level of *CYGB* mRNA in the aorta was decreased by 73% compared to normotensive rats. The expression level of CYGB protein was also decreased by 79% compared to normotensive rats (Fig. 1B, C). These data indicate that hypertension impaired *CYGB* expression at the gene and protein level.

Overexpression of *CYGB* improved VSMC migration

CYGB overexpression was observed in VSMCs (Fig. 2A, B); the expression of *CYGB* was 1.956±0.167 for VSMCs that overexpress *CYGB*, and 0.738±0.038 for the controls. Then, we explored the role of *CYGB* in cell migration by using VSMCs overexpressing *CYGB*. The VSMCs that overexpressed *CYGB* showed increased cell migration; the migration rate at 40 h was 14.335%±0.323% for VSMCs that overexpress *CYGB* compared to 10.292%±1.619% for the controls (Fig. 2C, 2D). As *MMP9* function is associated with degradation of the ECM and promotion of cell migration, we tested the expression of *MMP9*. We found that the expression

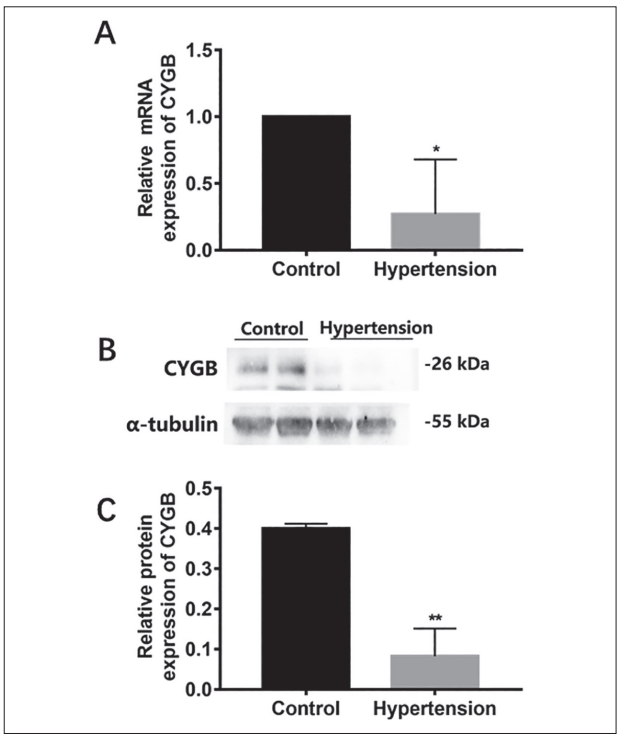

Fig. 1. *CYGB* expression in hypertension. **A** – qRT-PCR to detect decreased relative mRNA levels for *CYGB* gene expression in the aorta of hypertensive rats; *GAPDH* was used for internal normalization. **B** – Western blot analysis shows decreased *CYGB* protein expression in the hypertensive rat aorta; alpha tubulin was used as loading control. **C** – Histogram of the densitometric analysis of Western blots. All data are expressed as the mean±SD from three independent experiments. **P*<0.05; ***P*<0.01.

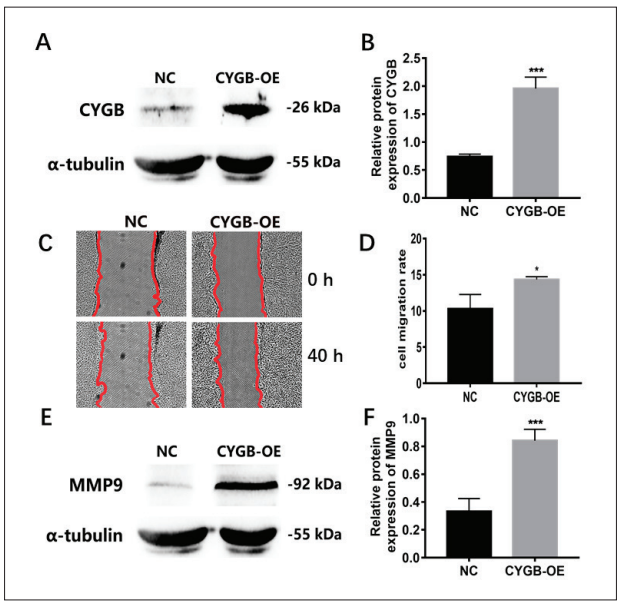

Fig. 2. The effect of *CYGB* overexpression on migration of VSMCs. **A, B** – *CYGB* overexpression was confirmed by Western blot; alpha tubulin was used as the loading control. **C** – Effect of *CYGB* overexpression on VSMCs migration analyzed by scratch assay. **D** – Quantitative measurement of cell migration rate. **E, F** – Increased MMP9 protein expression in VSMCs overexpressing *CYGB* was confirmed by Western blotting; alpha tubulin was used as the loading control. All data are expressed as the mean±SD from three independent experiments. **P*<0.05; ****P*<0.001.

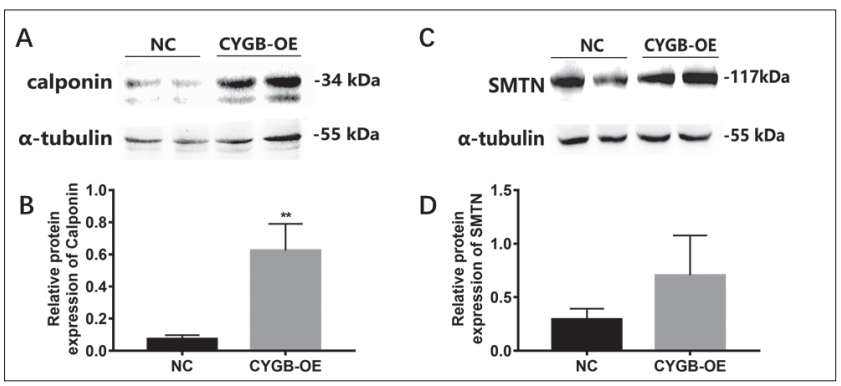

Fig. 3. The effect of *CYGB* overexpression on VSMC phenotype. **A, B** – Increased expression of calponin was detected by Western blot in VSMCs overexpressing *CYGB*; alpha tubulin was used as loading control. **C, D** – There was no significant difference of *SMTN* expression level between VSMCs overexpressing *CYGB* and control cells; alpha tubulin was used as the loading control. All data are expressed as the mean±SD from three independent experiments. ***P*<0.01.

of *MMP9* was 0.752±0.081 for VSMCs that overexpress *CYGB* compared to 0.287±0.168 for controls (Fig. 2E, F). These data suggest that overexpression of *CYGB* increased VSMC migration.

Effect of *CYGB* overexpression on the cell phenotype

Calponin is a troponin-T-like protein that interacts with F-actin and tropomyosin. Calponin is not only involved in the actin-associated system of regulation of smooth muscle contraction, but also is a differentiation marker of smooth muscle cells. We found that the expression of *calponin* in VSMCs that overexpressed *CYGB* was 0.624±0.136 and

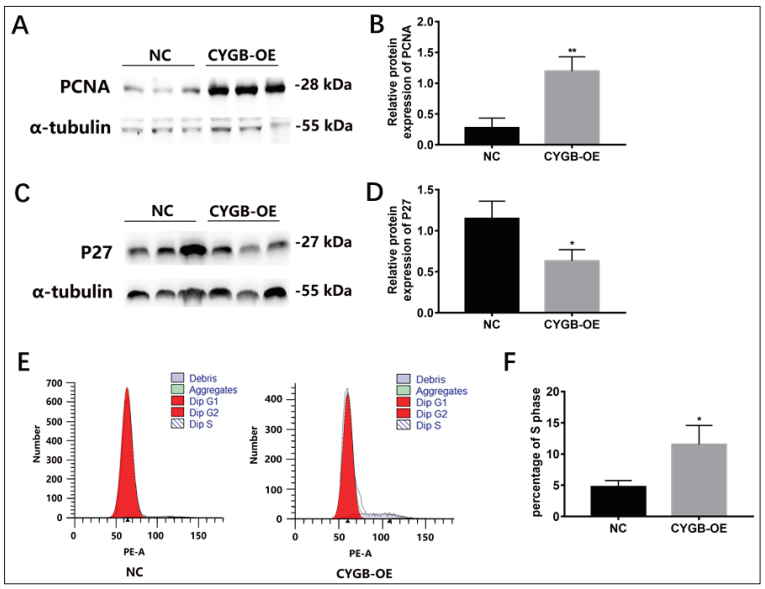

Fig. 4. The effect of *CYGB* overexpression on proliferation of VSMCs. **A, B** – Increased expression of *PCNA* was calculated by Western blotting in VSMCs overexpressing *CYGB*; alpha tubulin was used as the loading control. **C, D** – Decreased expression of *P27* was calculated by Western blotting in VSMCs overexpressing *CYGB*; alpha tubulin was used as the loading control. **E, F** – *CYGB* overexpression increased the number of VSMCs in the S phase of cell cycle as demonstrated by flow cytometry. All data are expressed as the mean±SD from three independent experiments. **P*<0.05, ***P*<0.01.

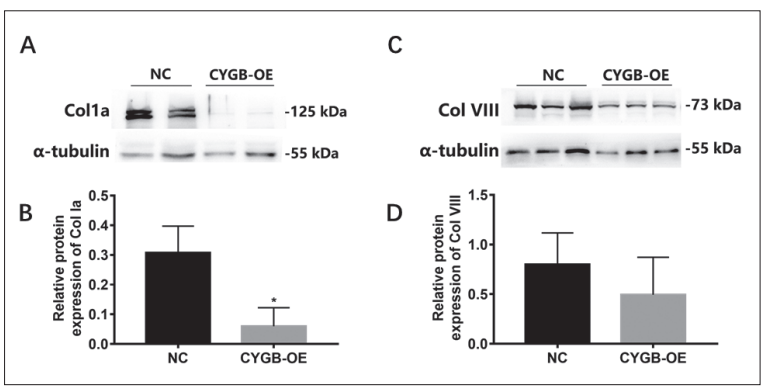

Fig. 5. The effect of CYGB overexpression on the expression of ECM components. **A, B** – Reduced protein expression of *Col Ia* was calculated by Western blotting in VSMCs overexpressing *CYGB*; alpha tubulin was used as the loading control. **C, D** – There was no significant difference of *Col VIII* expression between VSMCs overexpressing *CYGB* and control cells; alpha tubulin was used as the loading control. All data are expressed as the mean±SD from three independent experiments. **P*<0.05.

in controls it was 0.074±0.019 (Fig. 3A, B). However, overexpression of *CYGB* did not affect the expression of *SMTN*, which encodes for a cytoskeletal protein specific for smooth muscle cells in VSMCs (Fig. 3C, D). These data indicate that *CYGB* may be involved in VSMC phenotypic modulation.

Overexpression of *CYGB* promoted VSMC proliferation

We found that *PCNA*, a key factor in DNA replication and cell cycle regulation in *CYGB* overexpressing VSMCs was 1.196±0.189 and 0.275±0.129 in controls (Fig. 4A, B). However, the level of cyclin-dependent kinase inhibitor 1B (*p27*), an inhibitor of the cyclin dependent kinase involved in the regulation of the cell cycle, was significantly decreased compared to control VSMCs; the expression of *P27* in *CYGB*-overexpressing VSMCs was 0.633±0.110 and in controls it was 1.146±0.175 (Fig. 4C, D). The number of VSMCs overexpressing *CYGB* was 11.533±2.481 at the S phase, and in the controls it was 4.763±0.808 (Fig. 4E, F). These data indicate that overexpression of *CYGB* increased VSMC proliferation.

Overexpression of *CYGB* reduced expression of an ECM component

Collagen protein is an important component of the ECM. We tested the expression of *Col Ia* and *Col VIII*. As shown in Fig. 5, the level of *Col Ia* was 0.060±0.050 in *CYGB*-overexpressing VSMCs, and 0.307±0.073 in the controls (Fig. 5A, B). In contrast, the level of *Col VIII* was not significantly changed (Fig. 5C, D).

DISCUSSION

It was reported that the main pathological changes of hypertension are vascular reconstruction, VSMC proliferation, migration and ECM deposition [22]. Reports on the function of *CYGB* in VSMCs are rare. Only a few studies have shown that in a rat balloon angioplasty model, decreased expression of *CYGB* could prevent neointima formation. After complete ligation of the left common carotid, the *CYGB* knockout mice had no neointimal hyperplasia compared to wild-type mice [15]. Our study showed that *CYGB* was significantly decreased

in hypertensive rats compared to normotensive rats, thus we considered that *CYGB* may be involved in the pathogenesis of hypertension. To further study the effect of *CYGB* on VSMCs, *CYGB*-overexpressing cell lines were established by transfected lentivirus into VSMCs to explore the effect of *CYGB* on VSMCs.

The present study provides evidence for the molecular basis of *CYGB* contribution to vascular remodeling. VSMC migration is critical in many physiological and pathological processes [23, 24]. Herein, we show that overexpression of *CYGB* provided faster migration rates in the scratch monolayer cell wound assay compared to the control. We also found that VSMCs overexpressing *CYGB* had increased protein levels of *MMP9*, which enhanced the degradation of ECM and promoted cell migration. It has been reported that the increased *MMP9* expression in arterial walls was associated with hypertension [25].

Interestingly, the role of *CYGB* on other cells is opposite. For example, overexpression of *CYGB* in murine fibroblasts reduces cell migration by inhibiting PI-3k/mTOR signal pathways [26], and ectopic expression of *CYGB* inhibits the migration of breast cancer cells via downregulation of *GLUT1* and *HXK2* [27]. In contrast, the overexpression of *CYGB* augmented the migration of non-small cell lung cancer under hypoxia condition [28]. It appears that the effects of *CYGB* overexpression are cell-type specific.

VSMCs are the major cell type in normal arterial media. They have a low turnover and primarily perform contractile functions to control vascular tone. In disease states, VSMCs switch from the contractile phenotype to a synthetic phenotype, which leads to increased proliferation, migration, deposition of ECM and decreased levels of contractile marker proteins [29, 30]. Calponin is a contractile marker of VSMCs. In previous studies, VSMCs with decreased calponin expression had increased proliferation, while increased expression of calponin correlated with decreased VSMC proliferation [31]. In our study, we found that calponin expression was increased in VSMCs that overexpressed *CYGB* as compared to parental VSMCs.

Thus, overexpression of *CYGB* in VSMCs promoted the expression of not only the cell differentiation markers such as calponin, but also of the proliferation markers such as *PCNA*, a key factor in DNA replication and cell cycle regulation [32]. The mechanism to regulate the balance between differentiation and proliferation induced by the overexpression of *CYGB* in VSMCs remains to be elucidated.

Our results showed that not only *PCNA* [32], but *P27*, a cyclin-dependent kinase inhibitor, was decreased in *CYGB*-overexpressing VSMCs. The number of VSMCs overexpressing *CYGB* was significantly increased at the S phase of cell division compared to controls. These data suggest that *CYGB* may be involved in the regulation of VSMC proliferation during the phenotype switch. A previous study showed that the loss of *CYGB* prevented neointimal formation and caspase 3 activation after arterial injury [15]. Our study shows that *CYGB* was downregulated in the hypertensive rat, so we hypothesized that *CYGB* may have a protective effect and participate in feedback regulation *in vivo* [33].

Finally, *CYGB* is also involved in ECM remodeling. It is well established that *CYGB* modulates the expression of ECM components in various tissues and cells via distinct signaling pathways. For instance, the expression of *Col Ia* was decreased in *CYGB* transgenic mice [34], and *CYGB* also inhibited the synthesis of collagen in immortalized kidney fibroblasts [16]. In human tendon fibroblasts, *CYGB* overexpression decreases the synthesis of the ECM [35]. We found that the level of *Col Ia* was significantly decreased in *CYGB*-overexpressing VSMCs, suggesting that upregulation of *CYGB* in VSMCs suppresses the synthesis of *Col1a*. Taken together, our data indicate that *CYGB* plays an important role in the regulation of vascular remodeling, but the significance in physiology and pathology remains unclear and will need to be explored in future studies.

There are some limitations in this study. First, we only examined the effect of *CYGB* on the functions of VSMCs by the overexpression of *CYGB*, thus the effect of knockdown of *CYGB* in VSMCs remains unclear. Second, the effect of *CYGB* on VSMC function and vascular remodeling in hypertension was not examined in *in vivo* experiments. These topics need to be further investigated.

CONCLUSION

Our results show that the expression of *CYGB* was decreased in the aorta of the hypertensive rat.

Overexpression of *CYGB* promoted proliferation and migration of VSMCs and decreased *Col1a*. Ectopic expression of *CYGB* also modulated the expression of the VSMC phenotypic marker. The reason for this may be that *CYGB* plays a negative feedback regulation role *in vivo*. Due to its involvement in these processes, *CYGB* may have a potential role in the pathogenesis and vascular remodeling of hypertension.

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Conflict of interest disclosure: The authors have no conflict of interest.

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