Wnt5a inhibits hypoxia-induced pulmonary arterial smooth muscle cell proliferation by downregulation of β-catenin

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Hypoxia is an important trigger for pulmonary hypertension (PH). The typical pathophysiological changes of hypoxia-induced pulmonary hypertension (HPH) include pulmonary vascular constriction, pulmonary vascular remodeling (muscularization and thickening of pre-capillary pulmonary arteries, intimal proliferation, obliterator lesions), and thrombosis in situ (34, 35). It is generally believed that vasoconstriction is an early contributor to the disease process and that structural remodeling of the pulmonary vascular system becomes more important over time (11). Pulmonary vascular remodeling is characterized by uncontrolled and inappropriate proliferation of pulmonary arterial smooth muscle cells (PASMCs) (20). The mechanisms for pulmonary vascular remodeling and proliferation of PASMCs in PH remain unclear.

Wnts are a family of secreted glycoproteins with varying expression patterns and a range of functions (21). The Wnt signaling pathway is an ancient and evolutionarily conserved pathway, comprised, so far, of 19 known ligands, including Wnt1, 2, 3, 3a, 4, 5a, 5b, 6, 7a, 7b, 8a, 8b, 9a, 9b, 10a, 10b, 11, and Wnt16. Wnt signaling controls a broad variety of biological processes, including cell-fate specification, polarity, migration, and proliferation (41). Different Wnt ligands may have different effects on different cell types (7, 14, 38). Wnts trigger intracellular responses through various signaling pathways referred to as the following: 1) the β-catenin-dependent canonical pathway, 2) the noncanonical planar cell polarity pathway, and 3) the PKC/calmodulin kinase/nuclear factor of activated T cell-dependent pathway (10). In the β-catenin-dependent canonical pathway, upon binding of Wnts to its Frizzled (Fz) receptor, the cytosolic protein Disheveled (Dvl) inhibits glycogen synthase kinase-3, which allows accumulation of β-catenin and promotes its nuclear translocation, where it activates transcription of target genes together with transcription factors of the T-cell factor (TCF) family. A number of β-catenin-TCF target genes have been identified. Among them, Cyclin D1 is an important protein that plays a key role in cell cycle control and cell proliferation (36, 40).

Several lines of evidence have suggested that Wnt signaling may be involved in the pathogenesis of pulmonary hypertension. First, Wnt5a-mediated noncanonical signaling has been shown to regulate human endothelial cell proliferation and migration (1). This indicates that the Wnt signaling network has the potential to play an important role in the regulation of endothelial cell fate and, therefore, in vascular remodeling. Second, it has been demonstrated that, in idiopathic pulmonary arterial hypertension, canonical and noncanonical Wnt pathways can promote angiogenesis and inhibit vascular regression (5). Third, Wnt signaling is thought to regulate smooth muscle precursor development in the mouse lung (3) by inhibiting β-catenin/TCF signaling, which reduces vascular smooth muscle cell proliferation (32).

PULMONARY HYPERTENSION (PH) is a syndrome resulting from restricted flow through the pulmonary arterial circulation, which leads to pathological increases in pulmonary vascular resistance (PVR) and ultimately to right heart failure (26). The incidence of PH was 7.6 cases per million population, and the corresponding prevalence was about 26 cases per million population (29). Despite several technological and therapeutic advances over the past several years, PH remains a life-threatening, progressive disease with poor survival outcome.

Hypoxia is an important trigger for PH. The typical pathological changes of hypoxia-induced pulmonary hypertension (HPH) include pulmonary vascular constriction, pulmonary

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Despite extensive characterization of each signal transduction pathway in many Wnt-dependent processes, the contribution of Wnt signaling to the pathogenesis of HPH is not well elucidated. Thus the present study was designed to investigate the role of Wnt5a in hypoxia-induced proliferation of human PASMCs and its possible downstream pathway to determine the contribution of Wnt signaling to the development of pulmonary vascular remodeling in HPH. The results of this study will help to understand the novel strategies for PH treatment involving Wnt signaling.

**MATERIAL AND METHODS**

**Cell culture and preparation.** Human PASMCs from normal human subjects (Cascade Biologics Incorporated, Portland, OR) were cultured in tissue culture medium (Dulbecco’s Modified Eagle Medium, DMEM), supplemented with 100 μg/ml of penicillin, 100 IU/ml streptomycin, and 10% (vol/vol) fetal bovine serum (FBS) and maintained at 37°C in a humidified normoxia condition (21% O2-5% CO2, 74% N2). Cells were passaged (passages 4–8) after reaching confluency.

**Cell proliferation assay.** Cell proliferation was quantified by cell counting with a hemocytometer using 0.45% Trypan blue (Sigma-Aldrich) or methyl thiazolyl tetrazolium (MTT) assay (Sigma-Aldrich) for quantitative comparisons. All results were repeated at least three times.

**Real-time PCR was performed using a Mx3000P System (Stratagene, La Jolla, CA), and PCR reactions were performed in a 20-μl volume composing 0.25 μl purified cDNA, 10 μl Brilliant SYBR green QPCR Master Mix (Stratagene), 100 nM of primers, and 0.05 μl Rox reference dye and RNase-free distilled water. A 40-cycle PCR was carried out using the following conditions: pre-denaturation for 10 min at 95°C, denaturation for 30 s at 95°C, annealing for 1 min at 55°C, and elongation for 1 min at 72°C.**

**RT-PCR and real-time PCR.**

<table>
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<th><strong>Target Gene</strong></th>
<th><strong>Accession Number</strong></th>
<th><strong>Primer Sequence (location of the primers)</strong></th>
<th><strong>Product Size, bp</strong></th>
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<tr>
<td>Wnt5a</td>
<td>NM_003392.3</td>
<td>Sense: 5'-TGAGACGAGCGGAAAC-3' (3083–3097)</td>
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<tr>
<td></td>
<td></td>
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<tr>
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<td>NM_001904.3</td>
<td>Sense: 5'-CAAGGGTTGCTGATAGACG-3' (1586–1604)</td>
<td>190</td>
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<tr>
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</tr>
<tr>
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<td>Cyclin D1</td>
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<td>Antisense: 5'-TTAGAGTTGCTGATCGCAAG-3' (897–917)</td>
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<td></td>
<td>Antisense: 5'-TGGTTGGCGCGCCGGCAGACA-3' (703–725)</td>
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**Immunocytochemistry and confocal microscopy observation.** The human PASMCs grown on glass slides were washed in PBS, fixed immediately for 30 min at room temperature with 4% formaldehyde in Dulbecco’s PBS (D-PBS), blocked with blocking solution (2% BSA in D-PBS) for 15 min and incubated with 0.2% Triton X-100 in blocking buffer for 30 min at room temperature. Cells were incubated in D-PBS and stained with ethidium bromide. PCR product bands were visualized by ultraviolet light (Bio-Rad). Intensity values were measured by densitometric analysis with Quantitative One software (Bio-Rad), and normalized to the intensity values of β-actin for quantitative comparisons.

**Table 1. Primers used for PCR**

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**TCF, T-cell factor.**
with primary antibodies of β-catenin (1:100, Santa Cruz Biotechnology) overnight in a humid chamber at 4°C. After being washed with 0.2% Triton X-100 three times in blocking buffer, 5 min each, cells were incubated for 30 min at 37°C with the fluorescence-conjugated secondary antibodies (FITC-conjugated Affinity Pure Goat Anti-rabbit IgG, 1:100, Santa Cruz Biotechnology), and cell nuclei were stained with Hoechst 33258 (1:1,000, Sigma-Aldrich). After being mounted with Aqua-Mount (Biomedia, Foster City, CA), the images were observed at 1-μm step intervals over their entire z-axis using a ×100 oil-immersion objective.

siRNA preparation and treatment. siRNA oligonucleotides with two thymidine residues (dTdT) at the 3’ end of the sequence were purchased from GenePharma (GenePharma, Shanghai, China). The siRNA oligonucleotides were selected to correspond to the nucleotide sequence of 1932–1950: 5’-GGACACAGCAGCAAUUUGU-3’ for human β-catenin gene (Gene ID: 1499). The sequences of siβ-catenin RNA were as follows: sense: 5’-GGACACAGCAGCAAUUUGU-3’, anti-sense: 5’-ACAACACUCGUUGUCCCTT-3’. The sequences of negative control siRNA were as follows: sense: 5’-UUUCUGCAACGUGU-CAGUGTT-3’, anti-sense: 5’-ACGUGACAGUGUGUCAATT-3’. For human Cyclin D1 gene (Gene ID: 595), the sequences of siRNA (corresponding to 677–695: CCCGCAGAUUAUCAGAA) were as follows: sense: 5’-CCCAGACGAUUACUUGATT-3’, anti-sense: 5’-UUCAGAAGAUGACGCAGGTT-3’. The sequences of negative control for it were as follows: sense: 5’-UUUCGCAAACGUGU-CACGUTT-3’, anti-sense: 5’-ACGUGACAGUGUGUCAATT-3’. The sequences of siRNA/plasmids and the transfection reagent mixture) were added to the medium in a drop-wise manner and mixed gently by rocking the media back and forth. After 4–6 h, the cell culture medium was changed back to DMEM containing serum and antibiotics and incubated at 37°C for 48 h before proliferation assay, Western blot analysis, or PCR experiments. The fluorescently labeled siRNA (FAM-siRNA) was used to detect transfection efficiency.

Drugs and reagents. tmWnt5a (R&D Systems, Minneapolis) was dissolved in PBS (0.2% BSA in D-PBS) to form stock solution. β-Catenin plasmids and pcDNA3 plasmids were dissolved in deionized water to form the stock solution. siRNA (GenePharma) was dissolved in diethyl pyrocarbonate water to form the stock solution. MTT was dissolved in PBS to form stock solution.

Statistical analysis. Data were presented as means ± SE. Comparison between the groups of data was evaluated using the Student’s unpaired t-test. For multiple comparisons, one-way ANOVA was used with a Bonferroni post hoc test. A P value < 0.05 was considered statistically significant. All experiments were performed at least in three independent assays under similar conditions.
RESULTS

Gene expression of Wnt5a pathway molecules in human PASMCs. To establish the presence of the Wnt signaling pathway in human PASMCs, we first analyzed the gene expression profile of Wnt5a-related molecules in human PASMCs. As shown in Fig. 1, PCR amplification products were obtained with Wnt5a, Dvl1, β-catenin, TCF4, and Cyclin D1 primers. These results confirmed the expression of Wnt5a and the canonical Wnt downstream pathway members (β-catenin and Cyclin D1) in cultured human PASMCs.

Hypoxia-induced proliferation of human PASMCs. We next examined the mitogenic effect of hypoxia on human PASMCs. Compared with normoxia conditions (21% O2, 48 h), hypoxia (3% O2, 48 h) significantly increased proliferation of human PASMCs as determined by cell counting (Fig. 2A), MTT assay (Fig. 2B), and quantification of DAPI-stained nuclei (Fig. 2C), respectively.

Effect of hypoxia on the expression of Wnt5a pathway molecules. To test whether Wnt5a signaling is involved in the hypoxia-induced proliferation of human PASMCs, the expression of Wnt5a pathway molecules was examined after hypoxia exposure. As shown in Fig. 3, hypoxia (3% O2, 48 h) decreased Wnt5a expression at both mRNA (Fig. 3A) and protein level (Fig. 3B).

It has been reported that β-catenin accumulation and nuclear translocation is a key step for its role in cell cycle control, proliferation, and cell fate determination (24). Thus the effect of hypoxia on the expression level of β-catenin and its subcel-

Fig. 4. Hypoxia upregulated the expression of β-catenin and Cyclin D1. A: after human PASMCs were treated with DMEM (2% FBS) in normoxia (21% O2) or hypoxia (3% O2) for 48 h, the expression of β-catenin was detected by using real-time PCR (a) and Western blotting analysis (b) (n = 3). The molecular masses of β-catenin and β-actin are 92 kDa and 43 kDa. B: translocation of β-catenin into nucleus determined by confocal microscopy of immunofluorescence. The primary antibody of β-catenin was detected with fluorescence-conjugated secondary antibodies (FITC-conjugated Affinity Pure Goat Anti-rabbit IgG, green). Slides were counterstained with nuclei dye Hoechst 33258 (blue) (n = 3). C: translocation of β-catenin into nucleus determined by Western blotting for phospho-β-catenin (Ser-675) (n = 3), whose molecular masses is 92 kDa. D: expression level of Cyclin D1 was detected by real-time RT-PCR (a) and Western blotting (b) (n = 3), the molecular masses of Cyclin D1 is 38 kDa. The bar graph representing means ± SE data normalized to the amount of β-actin. *P < 0.05 vs. normoxia. **P < 0.01 vs. normoxia. ***P < 0.001 vs. normoxia.
lular localization were determined by using semiquantitative Western blot analysis and immunocytchemistry techniques. As shown in Fig. 4A, the mRNA and protein level of β-catenin significantly increased after hypoxia exposure. The distribution of β-catenin was found both in cytoplasm and cell nucleus under normoxia condition. Hypoxia induced upregulation of β-catenin in the cytoplasm and nucleus (Fig. 4B).

Phosphorylation of β-catenin at Ser-675 induces its accumulation in the nucleus and increases its transcriptional activity (39), so we further examined the phosphorylated β-catenin level under hypoxia condition and found that hypoxia upregulated the expression of phosphorylated β-catenin (Fig. 4C).

Cyclin D1, which plays a role in cell cycle control and proliferation, is an important β-catenin target gene. Thus we next determined the change of Cyclin D1 expression under hypoxia conditions. The results showed that the mRNA and protein level of Cyclin D1 in human PASMCs were upregulated after exposure to hypoxia for 48 h (Fig. 4D).

The above results suggest that hypoxia induced downregulation of Wnt5a but upregulated β-catenin and Cyclin D1 protein level as well as the nuclear translocation of β-catenin.

Hypoxia-induced human PASMC proliferation was dependent on the upregulation of β-catenin and cyclin D1. To investigate the role of Wnt pathway in hypoxia-induced human PASMC proliferation, β-catenin was knocked down in human PASMCs by transfecting siRNA targeting β-catenin. As shown in Fig. 5, transfection of β-catenin siRNA caused a reduction in β-catenin expression and the inhibition efficacy was about 56.2% at mRNA level (Fig. 5A) and 53.3% at protein level (Fig. 5B). The hypoxia-induced proliferation of human PASMCs was significantly inhibited by β-catenin siRNA transfection (Fig. 5C). The similar results were observed when Cyclin D1 was knocked down by siRNA (Fig. 6). The inhibition efficacy of Cyclin D1 siRNA transfection was about 46.5% at mRNA level (Fig. 6A) and 54.4% at protein level (Fig. 6B). These findings indicate that hypoxia-induced human PASMC proliferation is dependent on the upregulation of β-catenin and its target gene Cyclin D1.

Wnt5a inhibited hypoxia-induced human PASMC proliferation as well as the upregulation of β-catenin and cyclin D1. The above results suggest that the Wnt5a/β-catenin/Cyclin D1 pathway is involved in hypoxia-induced proliferation of human PASMCs. The inhibitory effect of hypoxia on Wnt5a expression indicates that the effect of Wnt5a on hypoxia-induced proliferation of human PASMCs may be inhibitory. To test this hypothesis, recombinant Wnt5a (rmWnt5a, 200 ng/ml) was used to treat human PASMCs under hypoxic conditions. After 48 h, the cell proliferation, expression level of β-catenin and Cyclin D1 as well as β-catenin nuclear translocation were measured. As shown in Fig. 7, rmWnt5a inhibited hypoxia-induced proliferation of human PASMCs (Fig. 7, A and B). Furthermore, this inhibitory effect was blocked by an anti-Wnt5a-antibody (2 μg/ml) (Fig. 7C). Hypoxia-induced upregulation of β-catenin and Cyclin D1 protein levels in human PASMCs were also decreased after treatment with rmWnt5a (Fig. 8A). Meanwhile, the hypoxia-induced β-catenin nuclear translocation was also blocked by rmWnt5a (Fig. 8, B and C). Taken together, these results suggest that rmWnt5a has an inhibitory effect on hypoxia-induced proliferation of human PASMCs, which may act through its suppression of β-catenin and Cyclin D1.

DISCUSSION

In this study, we have demonstrated that 1) hypoxia-induced proliferation of human PASMCs is accompanied by downregulation of Wnt5a expression, increased β-catenin and Cyclin D1 expression, as well as enhancement of β-catenin nuclear translocation, 2) β-catenin and its target gene Cyclin D1 play an important role in hypoxia-induced proliferation of human PASMCs.
PASMCs, and β) Wnt5a inhibits hypoxia-induced proliferation of human PASMCs through the β-catenin pathway. To our knowledge, this is the first report to demonstrate that the Wnt5a/β-catenin/Cyclin D1 pathway is involved in hypoxia-induced proliferation of human PASMCs.

As a member of Wnt family, Wnt5a has been seen as an oncogene because it promotes cell proliferation (17, 31). It has been reported that Wnt5a regulates human endothelial cell proliferation and migration through noncanonical signaling (1), whereas, in some other reports, Wnt5a has also been proposed to have antioncogenic property (15, 19). A recent report suggested that exogenous recombinant Wnt5a protein repressed JAR choriocarcinoma cell proliferation (30). Thus it is likely that whether Wnt5a has an oncogenic or tumor suppressive effect is highly dependent on the cell type and context in which the signaling occurs. Recent reports suggest that Wnt5a can play its role in β-catenin-dependent canonical Wnt signaling pathway. For example, Wnt5a acts as an antagonist of the Wnt/β-catenin pathway in mammary gland (33) and in colorectal cancer (43). HPH is a disease that is characterized by remodeling in HPH. Understanding this pathway will allow us to determine the contribution of Wnt5a to the development of pulmonary vascular remodeling in HPH.

The results of this experiments demonstrated that hypoxia-induced proliferation of human PASMCs was accompanied by the upregulation of Cyclin D1, β-catenin, and its nuclear translocation. It is known that β-catenin interacts with various transcriptional activators such as lymphoid enhancer-binding factor/TCF family members (25, 27), regulating cell proliferation. β-Catenin is kept at low level in the cytoplasm by the destruction complex, which is formed by the active serine-threonine kinase glycogen synthase kinase-3β, the adenomatous polyposis coli, and Axin. The β-catenin/TCF complex activates transcription of many different target genes such as Cyclin D1 (22). To determine whether β-catenin and Cyclin D1 play an active role in proliferation of human PASMCs, we examined proliferation of human PASMCs after manipulation of β-catenin and Cyclin D1 expression. We found that knockdown of β-catenin or Cyclin D1 inhibited hypoxia-induced proliferation of human PASMCs, whereas overexpression of β-catenin increased hypoxia-induced proliferation of human PASMCs.

The downregulation of Wnt5a induced by hypoxia in this experiment implied that Wnt5a may play an inhibitory role in hypoxia-induced proliferation of human PASMCs. To demonstrate this, human PASMCs were treated with rmWnt5a. Hypoxia-induced proliferation of human PASMCs, upregulation of β-catenin and Cyclin D1 as well as enhancement of β-catenin nuclear translocation were significantly inhibited by rmWnt5a. These results suggested that Wnt5a indeed has the

![Graph A](image1.png)

**Fig. 6.** Hypoxia-induced proliferation of human PASMCs was dependent on Cyclin D1. A: real-time RT-PCR (n = 3) results for Cyclin D1 expression after knockdown by siRNA oligomer. B: protein level of Cyclin D1 after knockdown by siRNA oligomer (n = 3). C: cell viability of each group was detected by MTT (n = 3). The bar graphs represent means ± SE, *P < 0.05 vs. N.C. ***P < 0.001 vs. N.C. ##P < 0.01 normoxia vs. hypoxia.

![Graph B](image2.png)

**Fig. 7.** The inhibitory effect of rmWnt5a on hypoxia-induced proliferation of human PASMCs. (N: normoxia, H: hypoxia, Ctrl: control, anti: anti-Wnt5a antibody). A: cell number counting under normoxia, hypoxia (3% O2, 48 h), and hypoxia plus rmWnt5a (n = 10). B: summarized data of DAPI staining for human PASMCs under normoxia, hypoxia, and hypoxia plus rmWnt5a (n = 6). The results are presented as means ± SE, **P < 0.01 vs. normoxia, #P < 0.05 vs. hypoxia. C: cell viability measured by MTT method under normoxia (a) and hypoxia (b) after the cells were treated with rmWnt5a or rmWnt5a antibody (n = 6). The results are presented as means ± SE, *P < 0.05 vs. control. ###P < 0.001 vs. rmWnt5a.
inhibitory effect on hypoxia-induced proliferation of human PASMCs and β-catenin maybe the downstream pathway.

To further clear up that the pivotal role of β-catenin in the inhibitory effect of rmWnt5a, β-catenin was overexpressed in human PASMCs. The inhibitory effect of Wnt5a on hypoxia-induced human PASMCs proliferation was significantly offset. Therefore, from the above results, it can be concluded that Wnt5a plays an inhibitory role in hypoxia-induced proliferation of human PASMCs through canonical Wnt/β-catenin pathways. Consistent with our results, some literatures have also reported that β-catenin signaling pathway was activated by hypoxia (4, 6, 8, 42).

Even though Wnt signal has been extensively studied in various fields including some lung diseases, including lung cancer (9), pulmonary fibrosis (2), and pulmonary hypertension (16). It is still a new member in the research of HPH. The results of this paper suggested that Wnt5a is involved in hypoxia-induced pulmonary arterial smooth muscle cell proliferation through β-catenin/Cyclin D1 pathway. However, whether it has any cross-talk with the classical signal such as Ca2+, which has been demonstrated to be responsible for the hypoxia-induced PASMC proliferation, is not clear.

In fact, there are already some reports that begin to express concern about the interaction between Wnt pathway and Ca2+

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**Fig. 8.** rmWnt5a decreased the expression of β-catenin and Cyclin D1 in human PASMCs exposed to hypoxia. Human PASMCs were cultured with DMEM (2% FBS) under normoxia (21% O2) or hypoxia (3% O2) with or without rmWnt5a (200 ng/ml), respectively. A: Western bolt results were displayed for β-catenin (92 kDa), Cyclin D1 (38 kDa), and β-actin (43 kDa) (n = 6). B: translocation of β-catenin into nucleus for normoxia, hypoxia, and hypoxia plus rmWnt5a was determined by confocal microscopy of immunofluorescence (n > 3). C: translocation of β-catenin into nucleus was determined by running Western blotting for phospho-β-catenin (Ser-675) (n = 3). Bar graph represents the data normalized to the amount of β-actin expressed as means ± SE. *P < 0.05, **P < 0.01 vs. normoxia. #P < 0.05 vs. hypoxia.
Ca\(^{2+}\) has been implicated as an important second messenger in the \(\beta\)-catenin-independent noncanonical Wnt pathway (13, 37). It has been reported that Wnt1 could effect transmembrane Ca\(^{2+}\) conductance through the L-type calcium channel (28), and Wnt5a-evoked calcium activity involves release from intracellular stores through IP3 receptors as well as calcium influx through TRP channels (18). In 2003, Ishitani et al. (12) had demonstrated that Wnt5a-stimulated Ca\(^{2+}\) was not investigated. Second, our researches are limited to in vitro experiments, and the effect of rmWnt5a on vascular remodeling in hypoxia-induced pulmonary hypertension animal model is needed to make the conclusion more solid. Third, the interaction between the Wnt pathway and Ca\(^{2+}\) signal should be elucidated in further experiments.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: X.-M.Y., L.W., J. Liu, J. Li, and W.W. performed experiments; X.-M.Y., L.W., and J. Li analyzed data; X.-M.Y. and J.W. drafted manuscript; L.W. and J.-F.L. prepared figures; J.-F.L. interpreted results of experiments; J.W. and C.W. conception and design of research; J.W. and C.W. edited and revised manuscript; J.W. and C.W. approved final version of manuscript.

REFERENCES


