Downregulation of Col1a1 induces differentiation in mouse spermatogonia

Sun-Hong Chen, Ding Li and Chen Xu

Col1a1 (one of the subunit of collagen type I) is a collagen, which belongs to a family of extracellular matrix (ECM) proteins that play an important role in cellular proliferation and differentiation. However, the role of Col1a1 in spermatogenesis, especially in the control of proliferation and differentiation of spermatogonial stem cells (SSCs), remains unknown. In this study, we explored effects of downregulation of Col1a1 on differentiation and proliferation of mouse spermatogonia. Loss-of-function study revealed that Oct4 and Ptfz, markers of SSC self-renewal, were significantly decreased, whereas the expression of c-kit and haprin, hallmarks of SSC differentiation, was enhanced after Col1a1 knockdown. Cell cycle analyses indicated that two-thirds of spermatogonia were arrested in S phase after Col1a1 knockdown. In vivo experiments, DNA injection and electroporation of the testes showed that spermatogonial self-renewal ability was impaired remarkably with the loss-of-function of Col1a1. Our data suggest that silencing of Col1a1 can suppress spermatogonia self-renewal and promote spermatogonia differentiation.

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Keywords: Col1a1; differentiation; extracellular matrix (ECM); spermatogenesis; spermatogonia

INTRODUCTION

Spermatogenesis is the process through which spermatogonial stem cells (SSCs) self-renew and differentiate into sperm. Spermatogenesis is composed of three particular phases: mitosis, meiosis and spermiogenesis.1 Previous studies have shown that spermatogenesis involves the detachment of spermatogonia from the basement membrane and their subsequent migration towards the lumen of seminiferous tubules.2–3 Although mechanisms underlying the maintenance and self-renewal of SSCs have been explored by many research groups,4–3 the signaling molecules that mediate the decision of SSCs to differentiate rather than self-renewal remain largely unknown. The balance between self-renewal and differentiation of SSCs must be tightly controlled to maintain normal homeostasis in the normal seminiferous epithelium. If self-renewal prevails, the seminiferous tubule will be stem cells only and tumors might occur.4 More differentiation than self-renewal of SSCs would result in Sertoli cells only in the seminiferous epithelium,2 suggesting that there must be a regulatory mechanism controlling the ratio between self-renewal and differentiation of SSCs.

Increasing evidences have shown that stem cell development requires a niche, a local microenvironment that mediates the proliferation and differentiation of stem cells in tissues or organs.6–9 The extracellular matrix (ECM) as a major niche element provides not only a scaffold for cellular support, but also a source of signals directing stem cell self-renewal, differentiation, cell adhesion and cell migration.5 The ECM is a compound of matrix molecules which are typically large glycoproteins, including collagens, fibronectins, laminins and proteoglycans.10 Collagen I, a major ECM component, may be involved in the regulation of cellular differentiation. Loss of adhesion to ECM components (e.g. collagen I) induces terminal differentiation of keratinocytes,11 and collagen I mRNA was down-regulated during mesenchymal stem cell differentiation into chondrocyte-like cells.12 Type I collagen is also a dedifferentiation marker for human nasal chondrocytes and fetal bovine chondrocytes, and can be switched from collagen II and collagen IX during the proliferation of chondrocytes.13,14 Procollagen I, a precursor of type I collagen, is exclusively expressed in mouse undifferentiated spermatogonial but absent in the differentiated male germ cells, suggesting that procollagen I is associated with maintenance of SSCs.15 However, the role of procollagen I in regulating SSC fate remains elusive. Two Col1a1 chains and one Col1a2 chain form procollagen I. The objective of this study was to elucidate the functional and developmental role of Col1a1 during spermatogenesis. To this end, we performed a loss-of-function study of Col1a1 in vitro to observe changes of molecular phenotype of SSCs. We then used an antisense strategy to knock down the expression of Col1a1 in SSCs purified from 6-day-old mice. Here, we show the influence of Col1a1 on the spermatogonia’s proliferation and differentiation.

MATERIALS AND METHODS

Ethics statement

All experiments were conducted following the Guide for Care and Use of Laboratory Animals (the ‘NIH Guide’). The protocols for the use of animals were approved by Department of laboratory animal sciences, Shanghai Jiao Tong University School of Medicine, with the permit number: SYXK/20080050.

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Plasmids and other reagents

The pDsRed2-1 and pAAV–IRES–hrGFP plasmids were gifts from Dr Lixin Feng (Shanghai Jiao tong University School of Medicine, China). Lipofection 2000 was purchased from Invitrogen (Carlsbad, CA, USA). T4 DNA ligase, restriction endonucleases Bgl II, Sal I, Not I and BamH I were purchased from New England Biolabs (Beijing, China). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Gibco BRL (Gaithersburg, MD, USA). PCR primers were synthesized by Shanghai Sangon Biological Engineering, Technology and Services (Shanghai, China). DNA sequencing was performed by Ding’an (Shanghai, China). Escherichia coli DH5α was grown in LB containing 50 mg l⁻¹ kanamycin.

Construction of a targeting vector both carrying mouse Stra8 promoter and antisense fragment of Col1a1

The first step was to construct pStra8-Dsred2-1, a plasmid containing 580 bp fragment of Stra8 (NM_009292) promoter. The −530/ +29 fragment amplified from Stra8 genomic DNA was inserted into the Bgl II site of pDsred2-1 (Table 1 and Figure 1a and 1b). The second step was to construct pStra8-Dsred2-1-antisense Col1a1, a plasmid containing an antisense fragment spanning the sequence of the first 1036 bp of Col1a1 (NM_007742) (Table 1 and Figure 1c). The fragment amplified from tests cDNA was inserted into the Bgl II/Sal I site of pDsred2-1, and the primers to amplify antisense of Col1a1 containing Sal I site in the sense primer and Bgl II site in antisense primer were designed respectively (Table 2). The 580 bp Stra8 promoter and pDsred2-1 (a kind of promoterless vector) could direct the antisense transcript of 580 bp fragment of Stra8 upstream region of the Col1a1 gene (NM_007742) (Table 1 and Figure 1d–1f). The plasmid pSP, containing all the fragments of pSPAPI but antisense of Col1a1, was served as a mock control.

Isolation of mouse spermatogonia

The decapsulated testes from 6-day-old male ICR mice were suspended in DMEM/F12 (Invitrogen) containing collagenase (1.5 mg ml⁻¹) and DNAase (1 µg ml⁻¹), and incubated at 34 °C for 15 min in a shaking water bath operated at 100 cycles per min. After two washes in DMEM/F12 medium, seminiferous tubule fragments mostly devoid of interstitial cells were incubated in DMEM/F12 medium containing collagenase (1.5 mg ml⁻¹), hyaluronidase (1.5 mg ml⁻¹) and trypsin (0.5 mg ml⁻¹) for 20–30 min using the method described above. The dispersed cells were washed twice with medium and through 80 µm and 40 µm nylon meshes respectively. The dispersed cells were incubated for 3–4 h, and then the Sertoli cells and peritubular myoid cells attached to the culture plates, while the spermatogonia still remained in suspension, which could be collected by centrifuging at 100 g for 5 min. Six-day-old mice were chosen in this study because only Sertoli cells and spermatogonia (As, Apair and Aaligned spermatogonia) are present in the seminiferous epithelium at this age.[16] The differential plating approach was used to separate spermatogonial populations from Sertoli cells and peritubular myoid cells, and the purity of the spermatogonia was >90%.

The spermatogenic capacity of the plasmid transfected cells were determined by flow cytometry, the transfected cells were stained with integrin β3 antibody conjugated with phycoerythrin, 10 µl for 10⁶ cells in 100 µl solution, and the ratio of the cells with both green and red fluorescence to the cells with green fluorescence was analyzed. Flow cytometry analysis was repeated at least three times.

Table 1 Sequences of the insert fragment in reconstructed plasmids

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Figure 1. Construction of the pSPAPI plasmid carrying mouse Stra8 promoter, antisense DNA fragment of Col1a1. The promoterless vector pDsred2-1 is a progenitor vector (a). The upstream region of the Stra8 gene (from –550 to +29) was amplified and cloned into the BglII site of the pDsred2-1 MCS (b), and thus, the vector could be transfected into spermatogonia specifically. A DNA fragment of Col1a1 (from +1 to +1036) containing enzyme site SalI in 5’ end and enzyme site BglII in 3’ end respectively was then inserted into BglII/SalI site of the plasmid (c). pAAV–IRES–hrGFP was digested with BamHI and NotI, and then the fragment containing FLAG, Ires and hrGFP (d) took the place of the fragment of pDsred2-1 between the BamHI and NotI sites (e). Finally, the recombinant vector was designated as pSPAPI (f). MCS, multiple cloning site.

Table 2. Oligonucleotide primers used for RT-PCR

<table>
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<tr>
<th>Name</th>
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R: 5’-GGAGATCTCGCCCTGACGAGTCAGTCACT-3’ (containing BglII) |
| Antisense sequence of procollagen I (from +1 to +1036) | F: 5’-TAATGCTCGACAGACTCATTCTTGAGACC-3’ (containing SalI)  
R: 5’-TGAAAGATCTAGCTTCACCCTTAGCACCAAC-3’ (containing BglII) |
| Stra8              | F: 5’-CGGATCCCTCAAAGCATCCTTCAACCT-3’  
R: 5’-GCAAGCTTTGGCGGCAGAGACAAAG-3’ |
| Oct4               | F: 5’-CACGAGTGAAAGCAACTCA-3’  
R: 5’-AGATGGTGCTCTGGATGACC-3’ |
| Plzf               | F: 5’-GACCTGGAATCACCTGCTTAGT-3’  
R: 5’-CTCTCGAGATCTGACTCAGCT-3’ |
| c-kit              | F: 5’-CCGGACGCAAATCTCTCTA-3’  
R: 5’-GCTCTCTGGCTGTCTCTGCT-3’ |
| Acrosin            | F: 5’-CGGAGTCTAACACAGCCACCT-3’  
R: 5’-GCATGAGTGAGAGCGAGTT-3’ |
| Haprin             | F: 5’-CCGAACATGAGACAGAGAG-3’  
R: 5’-AGCAGACTCTCGAGACATACC-3’ |
| Pcca               | F: 5’-GCAGGAGTACAGCTGTGTA-3’  
R: 5’-CAGACTATTCTCGCTCTTGAGTT-3’ |
| Col1a1             | F: 5’-GCCCATCAAGGATCTATG-3’  
R: 5’-AGCGGAAACATCGGCTGA-3’ |
| Gapdh              | F: 5’-AAGGCTATGACACAGATG-3’  
R: 5’-ACACATTGGGATGGAACA-3’ |
Cell culture and transfection

Mouse spermatogonia were cultured in DMEM/F12 (Invitrogen) supplemented with 10% fetal calf serum, penicillin (100 U ml⁻¹) and streptomycin (100 g ml⁻¹) at 37 °C in a humidified incubator with 5% CO₂. For the long-term maintenance of SSC potential, the isolated spermatogonia were cultured with Sertoli cells as feeder cells. Cells were counted and seeded onto 10-cm dishes with 10⁷ cells per dish. After 12 h of culture, cells were transfected with the recombinant vector pSPAPI using Lipofection 2000 reagent, according to the manufacturer’s manual. The transfected cells and supernatant were then harvested after 36 h. Vector pSP was used as a mock. After 36 h, the transfected primary spermatogonia with Sertoli cells were used for fluorescence-activated cell analysis sorting (FACS). Cell sorting was performed using a dual-laser fluorescence-activated cell sorter (FACS VantageSE; Becton Dickinson, San Jose, CA, USA). Cells showing green fluorescence were collected as positive cells.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the cultured cells using Trizol reagent (Invitrogen). The first-strand cDNA was synthesized using PrimeScript RT Reagent Kit (Takara). PCR was performed using specific primers (Table 2). House keeping gene, Gapdh, was used as an internal standard in all experiments. RT-PCR bands were quantified using Quantity One Software (Bio-Rad, Hercules, CA, USA), and the data were presented as mean±s.e.m. from three experiments. The mRNA transcript expressions of each gene were evaluated relative to the Gapdh expression in the individual samples and are presented as a ratio to that in controls.

Western blotting

Spermatogonia were transfected as described above and cell lysates were prepared in EBC buffer (50 mM Tris pH8, 170 mM NaCl, 0.5% NP40, 50 mM NaF) supplemented with protease inhibitors. Thirty micrograms of protein lysate per sample was denatured and fractionated by 10% SDS–PAGE. The separated proteins were transferred to a nitrocellulose membrane followed by blocking with 5% nonfat milk in TBS containing 0.1% Tween. Membranes were incubated for 2 h at room temperature with anti-procollagen I antibody (1 : 300) following which anti-rabbit Ig conjugated with horseradish peroxidase at a 1 : 2000 dilution was used. The signals were detected with the Western Blotting Luminescent Reagent (Millipore, Billerica, MA, USA).

Flow cytometry and analysis of cell ploidy

The transfected spermatogonia were subjected to FACS using a FACS VantageSE apparatus at 24, 36 and 48 h respectively post-transfection. We assessed the proportion of cellular ploidy of testicular cells by staining with propidium iodide (2 µg ml⁻¹) in the GFP-positive cells. Cells were harvested using trypsin/EDTA solution (0.05% trypsin in 0.5 mMol l⁻¹ EDTA) and resuspended in PBS separately. For detection of the cell ploidy, testicular cells were stained with propidium iodide at a final concentration of 2 µg ml⁻¹ for 5 min at room temperature. Cell cycle distribution was determined by flow cytometry. Thousands of events were recorded for each sample and all analyzed events were gated to remove debris and aggregates. Cell cycle analysis was repeated at least three times.

DNA injection and electroporation of the testis

ICR mice were used for the in vivo DNA electroporation. Male mice (n=5) at 20 dpp were anesthetized with Nembutal solution. Testes were pulled out from the abdominal cavity, and approximately 15 µl of plasmid DNA (10–15 µg) solution were injected into the rete testis using glass capillaries under a binocular microscope. Within each mouse, left testis was injected with solution containing pDsRed2-1, while the right one was injected with pSPAPI. Electric pulses were delivered with an Electrosquare Porator ECM 830 Electro Square Porator (BTX, San Diego, CA, USA). Testes were directly held between a pair of tweezer electrodes (BTX). Square electric pulses were applied three times, and again three times in the reverse direction at 50 V for 50 ms for each pulse.

Histological examination

In prepubertal mice, type A and type B spermatogonia are presented by day 8, 2 days later, meiotic prophase is initiated. The testes were fixed 2 days after being electroporated with Bouin’s solution, and were then embedded in paraffin preceded in two steps: dehydration and clearing. Paraffin blocks of testes were sectioned at a thickness of 5 µm. After being deparaffinized, the sections were stained with hematoxylin and eosin. Images were taken with a microscope (Olympus, Tokyo, Japan) equipped with a CCD camera. Histology was compared between electroporated and control testes. At least three sections were examined per testis. Spermatogonia, Sertoli cells and primary spermatocytes within five seminiferous tubules each section were counted, respectively.

Statistical analysis

Statistical differences were assessed with t-test. Data are presented as mean±s.e.m., and P values <0.05 were considered statically significant.

RESULTS

The Stra8 promoter used for directing spermatogonia-specific expression

To verify the cellular activity and specificity of the Stra8 promoter used, the primary cells (spermatogonia with Sertoli cells) after 36-h transfection with pSPAPI were analyzed for GFP expression using confocal laser scanning microscopy. Green fluorescence was observed only in spermatogonia (Figure 2), suggesting that the Stra8 promoter used specifically directed expression of the transgene (antisense Col1a1) in spermatogonia. However, only ~10% spermatogonia were transfected by recombinant plasmid (Figure 2). Under the microscope, Sertoli cells were fibroblast-like and attached to the culture plates, while spermatogonia were round in shape in the suspension or adhered slightly to the plates. Flow cytometry analysis revealed that ~93% of the GFP-positive cells were integrin-beta3 (marker for primordial germ cell) positive, suggesting that the green cells were indeed spermatogonia.

Levels of both Col1a1 mRNAs and protein were reduced in mouse spermatogonia after the plasmid transfection

RT-PCR and western blot analyses of mouse spermatogonia were performed 24 h and 48 h, respectively after the cells were transfected with pSPAPI, and the cells transfected with pSP served as a mock control. Transfected cells were sorted and then proteins were extracted. In these experiments, both Col1a1 mRNAs and protein was decreased significantly in the transfected spermatogonia (Figure 3), suggesting that the antisense transcripts of Col1a1 indeed suppressed the transcription of the Col1a1 in mouse spermatogonia.

RT-PCR analysis for evaluation of differentiation

To determine the effect of downexpression of Col1a1, mouse spermatogonia were transfected with the recombinant plasmid pSPAPI.
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Almost completely silenced by Oct4 stable, whereas significantly down regulated. Spermatogonia were transfected with pSPAPI or with pSP. Compared to the mock controls, levels of Col1a1 in spermatogonia were significantly downregulated after antisense Col1a1 knockdown assays (Figure 3). We also analyzed the expression of some differentiation markers of spermatogonia, including Oct4, Stra8, Pcna, Gfrα-1 and Plzf. Levels of Pcna and Gfrα-1 were downregulated slightly, Stra8 remained stable, whereas Oct4, a marker of self-renewal spermatogonia, was almost completely silenced by Col1a1 knockdown, and Plzf, a transcription factor for spermatogonial stem cell renewal, was downregulated in spermatogonia with knockdown of Col1a1 (Figure 4a and 4b). We also analyzed the expression of some differentiation markers of spermatogonia, and observed upregulation of c-kit and Haprin, markers for differentiating spermatogonia. However, we could not detect Acrosin (Figure 4c and 4d).

Cell cycle analyses in mouse spermatogonia after Col1a1 knockdown

The cell cycle phase distribution of the plasmid transfected mouse spermatogonia was determined by flow cytometry. A representative cell cycle phase distribution of spermatogonia transfected with pSP and pSPAPI was shown in Figure 5. After 24 h, the experimental group (spermatogonia transfected with pSPAPI) displayed 65.95% ± 3.35% in G1 phase, and 4.97% ± 2.41% in G2 phase, suggesting an increase in cells at S phase. Testes electroporated with pSPAPI (expressing antisense Col1a1) and pSP (control) were collected and analyzed 2 days after electroporation. Histological analyses showed that the numbers of spermatogonia was reduced significantly in the seminiferous tubule (Figure 6a–6d), while the histology of control testes was similar to that of untreated testes (Figure 6e–6h). The numbers of Sertoli cell did not differ (P > 0.05) between the Col1a1 knockdown and control testes. Interestingly, the number of spermatogonia in Col1a1 knockdown testes was about sevenfold fewer (P < 0.01) than that in mock testes. However, the number of primary spermatocyte in Col1a1 knockdown
testes was about twice (P<0.05) of the number seen in mock control testes (Figure 6i).

**DISCUSSION**

In this study, we investigated the potential role of Col1a1 in spermatogonial self-renewal or differentiation. Our results revealed that a reduction in Col1a1 mRNA and protein levels could lead to an imbalance between self-renewal and differentiation of spermatogonia, characteristic of suppressed self-renewal and accelerated differentiation. Therefore, Col1a1 appears to be an important ECM component in maintaining normal spermatogonial homeostasis during spermatogenesis.

It is believed that N-propeptide region of collagens is important for its function. For example, the N-propeptide region of procollagen IIA binds to TGF-β1 and BMP-2, suggesting that the NH2-propeptide of procollagen IIA function in the ECM distribution of bone morphogenetic proteins during endochondral bone formation. Moreover, the NH2-propeptide of the cartilage-characteristic collagen, such as type II B, PIIBNP, is released into the ECM prior to formation of the collagen fibrils, and when it is suppressed by small interfering RNA, adhesion is blocked. Therefore, we chose the fragment (from +1 to +1036 bp) encoding the N-terminal of Col1a1 for antisense-mediated gene knockdown study.

Stra8 is exclusively expressed in spermatogonia, rendering it a suitable promoter for directing transgene expression in all spermatogonia. Out data suggest that the 400 bp fragment from the promoter region of Stra8 is sufficient for directing gene expression in spermatogonia, which is consistent with a previous report. Indeed, Stra8 promoter has been used to isolate spermatogonia including SSCs from the mouse testes.

To demonstrate the functional importance of the Col1a1 on spermatogonial self-renewal and differentiation, several gene markers were chosen for further analysis. When Col1a1 levels were reduced, levels of markers for mouse spermatogonia and progenitors including Pena, Gfra-1, Oct4 and Stra8, were changed in different way. Interestingly, Oct4, a hallmark for mouse SSCs, was reduced to undetected level in spermatogonia with the Col1a1 knockdown (Figure 4), while levels of Gfra-1 was relatively lower compared to control cells and Pena and Stra8 change slightly. Pena is expressed in proliferating spermatogonia and spermatocytes, but not in non-proliferating spermatogonia or preleptotene spermatocyte. Stra8 is a vertebrate-specific gene whose expression is crucial for initiation of meiosis in both male and female germ cells and its overexpression is known to promote the in vitro differentiation of mouse embryonic stem cells into spermatogonial progenitors.

Our observations suggest that in response to Col1a1 suppression, the spermatogonial proliferation appeared to be suspended, while their differentiation was triggered. Supporting this notion, levels of c-kit, a hallmark for mouse differentiating spermatogonia, and Haprin, novel haploid germ cell-specific gene in the mouse potentially involved in the acrosome reaction, were upregulated. Acrosin is a marker of the acrosome in spermatids. It is a proteolytic enzyme that hydrolyses the zona pellucida (ZP) of the oocyte in the process of fertilization. Acrosin mRNA could not be detected in the transfected spermatogonia, suggesting that spermatogonia differentiated into early spermatocyte but not spermatid. Downregulation of Plzf, a transcription factor involved in spermatogonial stem cell renewal, is also supportive of the notion that Col1a1 suppression can inhibit proliferation, but enhance differentiation. This effect was confirmed in vivo by electroporation-based gene delivery of Col1a1 antisense mRNA-coding plasmids.

The seminiferous epithelium in the mammalian testis is segregated into the basal and the apical (adluminal) compartments by the
that the cells were transfected by plasmid pSPAPI or plasmid pSP, respectively.

differentiate into type B spermatogonia and primary preleptotene compartment to produce additional germ cells, some of which

blood-testis barrier. Mitosis occurs in SSCs residing in the basal compartment to produce additional germ cells, some of which
differentiate into type B spermatogonia and primary preleptotene spermatocytes. During their transit at the BTB, preleptotene spermatocytes differentiate into diplotene spermatocytes, and then enter metaphase I to undergo meiosis I and meiosis II which produces haploid spermatids in the apical compartment. Collagen is one of the most abundant ECM proteins in basement membrane, and the basement membrane and integrins act as anchorage for undifferentiated spermatogonia. It has been shown that the de novo synthesis of collagens is associated with tight junction assembly. So we hypothesize that even the slightly restructuring in the basement membrane or cell junctions would disrupt the spermatogonia in undifferentiated state. The loss of the Col1a1 in basement membrane might impair spermatogenesis, perhaps by disturbing cell-matrix interactions or by changing the structure of basement membrane and dynamics of cell junctions. Our data presented here also suggest that the balance of the self-renewal and differentiation in spermatogonia can be altered in response to Col1a1 suppression. Progression of the cell cycle is precisely controlled by checkpoints, the signaling networks including ERK1/2 pathway that allows cells to monitor successive events and ensure ordered cell proliferation and genomic stability. He et al. have showed that ERK1/2 pathway may be essential for the GDNF-induced G1/S transition in SSCs. GDNF signals through a multicomponent receptor complex comprised of Ret and Gfrα1. Our data have shown that Gfrα1 levels decreased slightly after Col1a1 knockdown, suggesting that the ERK1/2 pathway may have been affected, leading to reduced interactions between GDNF with Gfrα1, and cell cycle arrest at the S phase. But it is still unclear why the downregulation of Col1a1 could affect the Gfrα1 expression.

In summary, our data suggest that Col1a1, as a component of the SSC niche, may play an important role in the control of the balance between SSC self-renewal and differentiation. Further study using conditional Col1a1 knockout mice is needed for unequivocally establish such a role of Col1a1.

AUTHOR CONTRIBUTIONS

SHC designed and performed the study, wrote the manuscript. DL performed the study. CX designed the study and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors have no competing financial interests to declare.

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