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ORIGINAL ARTICLE

Male Fertility

# NODAL secreted by male germ cells regulates the proliferation and function of human Sertoli cells from obstructive azoospermia and nonobstructive azoospermia patients

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This study was designed to explore the regulatory effects of male germ cell secreting factor NODAL on Sertoli cell fate decisions from obstructive azoospermia (OA) and nonobstructive azoospermia (NOA) patients. Human Sertoli cells and male germ cells were isolated using two-step enzymatic digestion and SATPUT from testes of azoospermia patients. Expression of NODAL and its multiple receptors in human Sertoli cells and male germ cells were characterized by reverse transcription-polymerase chain reaction (RT-PCR) and immunochemistry. Human recombinant NODAL and its receptor inhibitor SB431542 were employed to probe their effect on the proliferation of Sertoli cells using the CCK-8 assay. Quantitative PCR and Western blots were utilized to assess the expression of Sertoli cell functional genes and proteins. NODAL was found to be expressed in male germ cells but not in Sertoli cells, whereas its receptors ALK4, ALK7, and ACTR-IIB were detected in Sertoli cells and germ cells, suggesting that NODAL plays a regulatory role in Sertoli cells and germ cells via a paracrine and autocrine pathway, respectively. Human recombinant NODAL could promote the proliferation of human Sertoli cells. The expression of cell cycle regulators, including CYCLIN A, CYCLIN D1 and CYCLIN E, was not remarkably affected by NODAL signaling. NODAL enhanced the expression of essential growth factors, including GDNF, SCF, and BMP4, whereas SB431542 decreased their levels. There was not homogeneity of genes changes by NODAL treatment in Sertoli cells from OA and Sertoli cell-only syndrome (SCO) patients. Collectively, this study demonstrates that NODAL produced by human male germ cells regulates proliferation and numerous gene expression of Sertoli cells.

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## INTRODUCTION

Infertility is a common reproductive disorder, and male factors account for about 40% of infertility in the world.<sup>1</sup> Azoospermia, including OA and NOA, is a major cause of male infertility. OA patients are caused by the obstruction of the reproductive duct and they usually have normal spermatogenesis. In contrast, NOA is diagnosed as the dysfunction of spermatogenesis, which accounts for 60% of azoospermic men. SCO is a kind of NOA with the severe impairment of spermatogenesis, as diagnosed by the testicular biopsy displaying that seminiferous tubules are lined by only Sertoli cells, with complete depletion of male germ cells.<sup>2</sup> To the best of our knowledge, the molecular mechanisms underlying SCO have not been elucidated.

As the main supporting cells, Sertoli cells produce numerous growth factors that play crucial roles in regulating germ cell development and spermatogenesis. It has been demonstrated that Sertoli cells secrete glial cell line-derived neurotrophic factor (GDNF), stem cell factor (SCF),

and bone morphogenic protein 4 (BMP4) to regulate spermatogonial stem cell (SSC) self-renewal and differentiation.<sup>3–7</sup> It has been suggested that abnormal growth factor secretion from Sertoli cells would result in the dysfunctions of spermatogenesis, which may eventually cause azoospermia in humans.<sup>8,9</sup>

Despite limited information available, male germ cells could also secrete growth factors which might be crucial for maintaining Sertoli cell functions, including their proteins' production and blood testis barrier (BTB) integrity.<sup>10</sup> It has been reported that constitutive expression of IL-1a in Sertoli cells is dependent on the interaction with male germ cells.<sup>11</sup> In addition, as germ cells differentiate, their gene and protein expression and localizations are different, resulting in distinct cellular interactions.<sup>10</sup> It has been demonstrated that basic fibroblast growth factor (bFGF) is derived from pachytene spermatocytes and it could stimulate transferring expression in Sertoli cells.<sup>12</sup> Nerve growth factor (NGF), which is secreted by round spermatids, has been found to

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be involved in regulating Sertoli cells viability.<sup>12</sup> Growth differentiation factor 9 (GDF9) is specifically produced by round spermatids and it could inhibit Sertoli cells' tight junction integrity and promote inhibin B secretion in rats.<sup>13</sup> Thus, it has been proposed that male germ cells may exert various biological effects on Sertoli cell functions, contributing to create the appropriate spermatogenesis niche. However, whether there are specific growth factors produced by male germ cells to control the fate determinations and function of Sertoli cells in human remain to be defined.

NODAL is a member of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily, which has been considered to be crucial for several biological process, including embryogenesis, tissue morphogenesis, and carcinogenesis.<sup>14,15</sup> NODAL protein is composed of 347 amino acids in total length, including the signal peptide (1–26), the propeptide (27–237) and the main chain (238–347). The secondary structure of the main chain contains three TURN, three Beta strand and two Helix structures.<sup>16</sup> NODAL exerts its biological effect via binding to its multiple receptors, including ALK4, ALK7, and ACTR-IIB.<sup>17</sup> NODAL has been proved to maintain the pluripotency of embryonic stem cells (ESCs) and inhibit their differentiation.<sup>18</sup> NODAL is also required for endoderm and mesoderm formation and left-right axis specification. It has been reported that NODAL plays crucial roles in the development of heart, breast, and pancreas.<sup>19</sup> NODAL regulates cancer progression in many cases, e.g., prostate cancer, breast cancer, melanoma, and glioma.<sup>20</sup> In addition to these critical functions mentioned above, several lines of evidence have demonstrated that NODAL signaling plays important roles in mammalian reproduction. NODAL signaling has been found to be active in mouse XY germ cells during gonadal development. Disruption of NODAL signaling would decrease germline stem cell pluripotency and early XY germ cell differentiation.<sup>21</sup> We have demonstrated that NODAL promotes mouse SSC proliferation through Smad2/3 and *Oct-4* activation via an autocrine pathway.<sup>17</sup> However, it is still unknown whether NODAL signaling is involved in human Sertoli cell fate decision and function regulation.

In this study, we examined the expression, function, and signaling pathway of NODAL in human Sertoli cells. We demonstrated that NODAL was expressed in male germ cells, but not in Sertoli cells, whereas its receptors ALK4, ALK7, and ACTR-IIB were detected in Sertoli cells and germ cells, implicating that NODAL plays regulatory roles in human Sertoli cells via a paracrine manner. Furthermore, we found that NODAL could regulate the proliferation and functional gene expression of human Sertoli cells. The study thus illustrates the interaction or crosstalk between male germ cells and human Sertoli cells and it shed a novel insight into the mechanism underlying the niche of human testis.

## MATERIALS AND METHODS

### *Procurement of testicular biopsies from OA patients with normal spermatogenesis and SCO patients*

Testicular biopsies were obtained from azoospermia patients who underwent microdissection TESE (MD-TESE) at Ren Ji Hospital affiliated to Shanghai Jiao Tong University School of Medicine. Patients with OA were caused by inflammation and vasoligation, but not by congenital absence of the vas deferens (CBAVD) or other diseases including cancer. Patients with SCO were confirmed by histological analysis, and patients with reproductive congenital disease, e.g., Klinefelter syndrome, genomic AZF deletions, or other diseases, including cancer, were excluded from this study. Twenty OA

patients and SCO patients were selected in this study. This study was approved by the Institutional Ethical Review Committee of Ren Ji Hospital (license number of ethics statement: 2012-01), Shanghai Jiao Tong University School of Medicine, and an informed consent of testis tissues for research only was obtained from the donors.

### *Isolation and culture of human Sertoli cells from OA and SCO patients*

Testicular biopsies obtained from OA and SCO patients were washed 3 times aseptically in DMEM/F12 (Gibco, Grand Island, NY, USA) containing antibiotic with penicillin and streptomycin (Gibco, Grand Island, NY, USA). Sertoli cells were isolated from human testis biopsies using a two-step enzyme digestion as previously described.<sup>22</sup> Briefly, testicular tissues were first digested with collagenase type IV (2 mg ml<sup>-1</sup>, Gibco, Grand Island, NY, USA) and DNase I (1  $\mu$ g  $\mu$ l<sup>-1</sup>, Sigma) in DMEM/F-12 at 34°C for 10 min. After extensive washes to remove the interstitial cells, the seminiferous tubules were then digested with DMEM/F12 containing collagenase type IV (2 mg ml<sup>-1</sup>, Gibco, Grand Island, NY, USA), hyaluronidase (2.5 mg ml<sup>-1</sup>, Sigma), trypsin (2 mg ml<sup>-1</sup>, Sigma), and DNase I (10  $\mu$ g  $\mu$ l<sup>-1</sup>, Sigma) at 34°C for 15 min. The single cells suspension was seeded into culture plates at a density of approximately  $2 \times 10^5$  cm<sup>-2</sup> in DMEM/F-12 supplemented with 10% FBS (Gibco, Grand Island, NY, USA) and incubated at 34°C in 5% CO<sub>2</sub> for 3 h. After incubation, the media containing male germ cells were removed, and Sertoli cells attached to the plates and were cultured with the DMEM/F12 medium containing 10% FBS which was changed every 24 h. The cells were passaged using 0.25% trypsin when cells reached 70%–80% confluence. Human Sertoli cells were identified by reverse transcription (RT)-PCR and immunocytochemistry with anti-GATA4 and WT1 (Santa Cruz) as described below.

To detect the expression of human Sertoli cell genes and proteins, the cells were seeded in 6-well culture plates at a density of approximately  $2 \times 10^5$  cm<sup>-2</sup> with DMEM/F-12 containing 10% FBS. The cells were starved in serum-free DMEM/F12 for 24 h and treated without or with 20  $\mu$ mol l<sup>-1</sup> SB431542, a specific inhibitor for receptors ALK4/5/7, for 30 min, and 100 ng ml<sup>-1</sup> human recombinant NODAL (R and D System, Minneapolis, MN, USA) was added to the medium. After 24 h and 48 h of culture, cells were collected for RNA and protein extractions.

### *The isolation of human male germ cells*

Human spermatogonia, pachytene spermatocytes, and round spermatids were isolated from OA patients' testes. As described above, after Sertoli cells attached to the plates, the cells suspension containing abundant human germ cells were collected by centrifuging at 1000 rpm for 5 min. The cells were resuspended in 0.5% BSA solution and filtered through a 70  $\mu$ m mesh to remove cell aggregates. STAPUT apparatus was adopted to isolate human spermatogonia, pachytene spermatocytes, and round spermatids as previously described.<sup>23–29</sup> Briefly, a linear gradient in STAPUT apparatus was generated from 300 ml of 2% BSA and 300 ml of 4% BSA solutions to the corresponding reservoirs. Fifty milliliters of 0.5% BSA medium were loaded in the glass loading tube and let it go into the sedimentation chamber. Twenty-five milliliters of cells suspension were loaded, and the stirrer started under the tube and the cells went into the sedimentation chamber (~5–10 min). The stirrer started under the 2% BSA solution and the artery clips were removed so that both BSA solutions could flow to the chamber (~30 min). The artery clips were replaced and the stirrers were closed. The gradient was formed after another 3 h of sedimentation. The SP-180 cell sedimentation chamber was unloaded from the bottom in 15 ml fraction. In order to ensure the different cell

types, the first fraction collected was designated as 1 and the remainder was numbered through to 45. The cells in each fraction were collected by centrifugation at 1000 rpm for 5 min. The cells were examined under a microscope to assess cellular status and to identify the cell types. The cells with similar size and morphology were pooled. Human spermatogonia, pachytene spermatocytes, and round spermatids were identified by RT-PCR and immunocytochemistry with specific antibodies as described below.

#### Cell proliferation assay

The human Sertoli cells from OA and SCO patients were seeded in 96-well culture plates with DMEM/F-12 containing 10% FBS. The cells were starved in serum-free DMEM/F-12 for 24 h and treated with 50 ng ml<sup>-1</sup>, 100 ng ml<sup>-1</sup>, or 200 ng ml<sup>-1</sup> human recombinant NODAL. After 1 day to 5 days of culture, CCK-8 medium (Dojindo) was added to the cells. The optical density (OD) for each well was measured at 450 nm using microplate reader (Bio-Rad Model 550). The experiments were repeated 4 times in both OA and SCO groups.

#### RNA extraction and RT-PCR, and quantitative real-time PCR

Total RNA was extracted from human Sertoli cells and male germ cells using Trizol reagent (Invitrogen). RNA was reverse-transcribed into cDNA using oligo (dT) priming and M-MLV reverse transcriptase. PCR was performed to detect the expression of Sertoli cell and male germ cell specific genes, including *GATA4* (GATA binding protein 4), *WT1* (Wilms tumor 1), *ABP* (androgen binding protein receptor), *FSHR* (follicle stimulating hormone receptor), *VASA* (DDX4 DEAD (Asp-Glu-Ala-Asp) box polypeptide 4), *GFRA1* (glial cell line-derived neurotrophic factor family receptor alpha 1), *RET* (ret proto-oncogene), *UCHL1* (ubiquitin carboxyl-terminal esterase L1), *SCP1* (synaptonemal complex protein 1), *SCP3* (synaptonemal complex protein 3), *PRM1* (protamine 1), *PRM2* (protamine 2), *TP1* (transition protein 1), and *TP2* (transition protein 2). Additionally, *HSD3B* (hydroxy-delta-5-steroid dehydrogenase, 3 beta), *CYP17A1* (cytochrome P450, family 17, subfamily A, polypeptide 1), *SMA* (smooth muscle actin), *MYH11* (myosin, heavy chain 11, smooth muscle), *CD34*, and *CD105* were selected to assess if Leydig cells, peritubular cells, and mesenchymal cells existed in isolated human Sertoli cells. Real-time PCR reactions were carried out to detect the expression of Sertoli cell functional genes, including *GDNF* (glial cell line-derived neurotrophic factor), *SCF* (stem cell factor), and *BMP4* (bone morphogenetic protein 4),<sup>30</sup> using a Thermal Cycler Dice™ Real-time System (Takara) and the SYBR Premix Ex Taq™ reagents kit (Takara). The primers of these genes were listed in **Supplementary Table 1**. The experiments were repeated 4 times in both OA and SCO groups.

#### Histological examination

The testicular tissues were fixed in Bouin's solution overnight, followed by being embedded in paraffin and sectioned at 6 μm thickness. The sections were stained with hematoxylin and eosin (H&E) and observed under a microscope for the structure of seminiferous tubules.

#### Immunocytochemistry

Human Sertoli cells were seeded on cell culture plates or coverslips at a density of approximately 2 × 10<sup>5</sup> cm<sup>-2</sup>. The Sertoli cells were digested or directly fixed on coverslips in 4% paraformaldehyde (PFA) when they reached 70%~80% of confluence. Human freshly isolated spermatogonia, pachytene spermatocytes, and round spermatids were fixed in 4% PFA. The Sertoli cells on the coverslips were used to detect *GATA4*, *WT1* and *VIMENTIN* using immunocytochemistry. The

remaining testicular cells were used to detect proteins using antibodies against *GFRA1*, *PIWIL2*, *ACROSIN*, *VASA*, *NODAL*, *ALK4*, *ALK7* and *ACTR-IIB* using immunocytochemistry. In regard to testicular tissue *in situ* staining, testicular biopsies obtained from OA and SCO patients were washed 3 times in phosphate-buffered saline (PBS) and were immediately fixed with 4% PFA for 24 h. The PFA-fixed tissues were embedded and sectioned at 8 μm thickness. The sections were used to detect expression of *VASA*, *NODAL*, *ALK4*, *ALK7*, and *ACTR-IIB* using antibodies by immunohistochemistry. Briefly, the cells and the tissue sections were permeabilized with 0.4% Triton/X-100 in PBS. Blocking was performed in 1% bovine serum albumin (BSA) for 1 h prior to incubation with primary antibodies. The cells and slides were incubated with primary antibodies overnight at 4°C, followed by Alexa Fluor 555-labeled secondary antibody and/or Alexa Fluor 488-labeled secondary antibody for 2 h. DAPI (4', 6'-Diamidino-2-phenylindole) was used to label cell nuclei. Replacement of primary antibodies with isotype IgGs to secondary antibody species was used as negative controls. The rest of the procedure was the same as others of the experiment. The information of the primary antibodies was provided in **Supplementary Table 2**.

#### Meiotic spread assays

Meiotic spread assays were performed to determine the identity of freshly isolated pachytene spermatocytes from OA patients by STA-PUT velocity sedimentation. Briefly, cells were lysed by a hypotonic solution and spread evenly over slides layered with 1% PFA and 0.15% Triton X-100. Slides were dried for 24 h at room temperature in a humid chamber. The cells were treated with 0.04% photoflo for 5 min and blocked with 4% normal serum. Double staining was performed in cells incubated with primary antibodies, including *SCP3* (Abcam), *CREST* (Immunovision), and *MLH1* (Abcam) overnight at 37°C in a humid chamber. Alexa Fluor 555-labeled secondary antibody, Alexa 488-labeled secondary antibody, and AMCA-conjugated AffiniPure Donkey Anti-Human IgG(H+L) were used as the secondary antibodies and incubated for 90 min at 37°C. Cells were washed 3 times with TBS and treated with antifade (vector laboratories), and images were captured with a fluorescence microscope (Leica). The information of the primary antibodies was provided in **Supplementary Table 2**.

#### Western blots

Human Sertoli cells were lysed with RIPA buffer (Santa Cruz) for 30 min on ice. After 30 min lysis on ice, cell lysates were cleared by centrifugation at 12 000 g, and the concentration of protein was measured using the bicinchoninic acid Protein Assay Kit. Thirty micrograms of cell lysate from each sample were used for SDS-PAGE (Bio-Rad Laboratories, Richmond, CA, USA), and Western blots were performed according to the protocol described previously.<sup>30</sup> Primary antibodies included *CYCLIN A1* (Santa Cruz, CA, USA), *CYCLIN D1* (Santa Cruz, CA, USA) and *CYCLIN E* (Santa Cruz, CA, USA). The blots were detected by chemiluminescence (Chemi-Doc XRS, Bio-Rad, CA, USA) and IDV was calculated by comparing the signals of target proteins with that of housekeeper *ACTB*. The experiments were repeated 4 times in both OA and SCO groups. The information of the primary antibodies was provided in **Supplementary Table 2**.

#### Statistical analysis

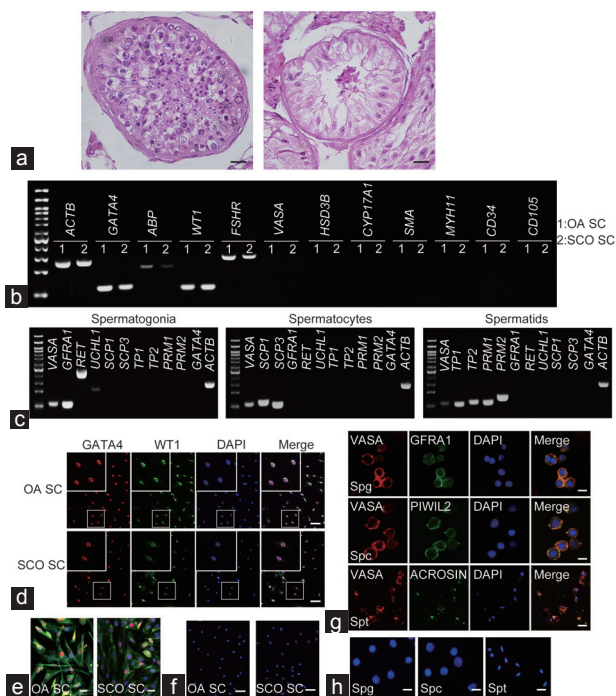
GraphPad Prism 5 (Version 5.01, GraphPad Software, Inc. CA, USA) was employed for statistical analysis. All data were presented as mean ± s.d., and statistical differences (*P* < 0.05) among groups were determined using the analysis of variance (ANOVA) and a paired *t*-test.



**RESULTS**

**Identification of human Sertoli cells and male germ cells**

Histological examination showed that Sertoli cells were located regularly on the basement membrane, forming seminiferous tubules in testis. As for testis from OA patients, there were male germ cells ranging from spermatogonia to spermatids within the seminiferous tubules. However, in the testis from SCO patients, only Sertoli cells were located along the basement membrane, with germ cells complete absence (Figure 1a). Moreover, it should be noted that the seminiferous epithelium integrity of testes from OA patients was unequal to that of normal testes.



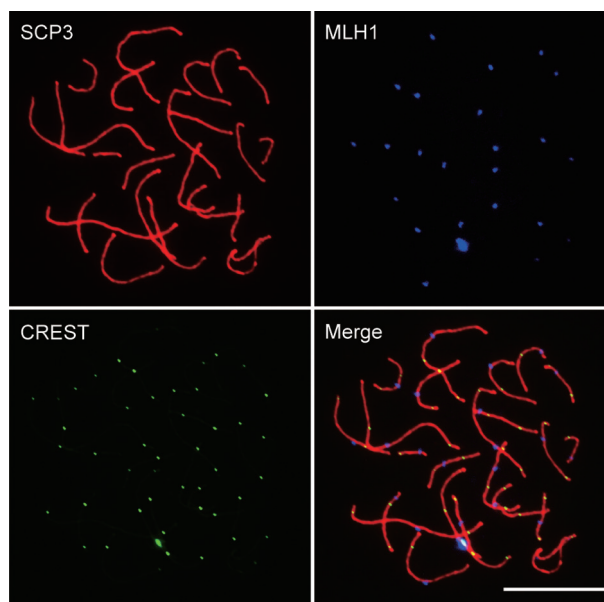
**Figure 1:** Morphology of testis from OA and SCO patients and identification of the isolated human Sertoli cells, spermatogonia, spermatocytes, and spermatids. (a): H&E staining illustrated the morphology of testicular tissues from OA (left panel) and SCO patients (right panel). Histological examination showed that seminiferous tubule from SCO testis had a reduced diameter, with only Sertoli cells along the basement membrane, compared with that of OA. Scale bars = 10  $\mu$ m. (b): The human Sertoli cells isolated from OA and SCO patient testes expressed transcripts of *GATA4*, *ABP*, *WT1*, and *FSHR* but no transcripts of *VASA*, *HSD3B*, *CYP17A1*, *SMA*, *MYH11*, *CD34*, and *CD105*. 1 and 2 represents OA Sertoli cells and SCO Sertoli cells, respectively. (c): RT-PCR showed that the expression of specific makers in isolated human spermatogonia (*GFRA1*, *RET*, and *UCHL1*), spermatocytes (*SCP1* and *SCP3*), and spermatids (*PRM1*, *PRM2*, *TP1*, and *TP2*). (d): Immunocytochemistry revealed that both *GATA4* (red fluorescence) and *WT1* (green fluorescence) were expressed in human Sertoli cells isolated from OA and SCO patients testes. The enlarged images were shown in the up-left of the pictures. Scale bar = 10  $\mu$ m. (e): Immunocytochemistry displayed the co-expressions of *GATA4* (red fluorescence) and *VIMENTIN* (green fluorescence) in human Sertoli cells isolated from OA and SCO patient testes. Scale bars = 10  $\mu$ m. (f): Negative control staining of immunocytochemistry of human Sertoli cells without primary antibody. Scale bars = 10  $\mu$ m. (g): Immunocytochemistry displayed the co-expressions of *GFRA1* (green fluorescence), *PIWIL2* (green fluorescence), and *ACROSIN* (green fluorescence) with *VASA* (Red fluorescence) in isolated germ cells. (h): Negative control staining of immunocytochemistry of the human germ cells without primary antibody. Scale bars = 10  $\mu$ m. Notes – OA: obstructive azoospermia, SCO: Sertoli cell-only syndrome, SC: Sertoli cells; Spg: spermatogonia; Spc: spermatocytes; Spt: spermatids. Passage 2 Sertoli cells were used in this experiment.

Sertoli cells from patients with OA and SCO were isolated by a two-step enzymatic digestion and followed by differential plating. Human spermatogonia, spermatocytes, and spermatids were further isolated by STAPUT approach. The isolated human Sertoli cells and male germ cells were identified through their specific markers. RT-PCR showed that the isolated cells expressed the transcripts of genes for Sertoli cells, including *GATA4*, *WT1*, *ABP*, and *FSHR* (Figure 1b), suggesting that these cells are Sertoli cells in phenotype. In addition, *HSD3B*, *CYP17A1*, *SMA*, *MYH11*, *CD34*, and *CD105* were hardly detected using RT-PCR, indicating that there was no contamination of Leydig cells, peritubular cells, or mesenchymal cells in the isolated Sertoli cells. The isolated germ cells expressed the genes specific for spermatogonia, including *GFRA1*, *RET*, and *UCHL1*, or *SCP1* and *SCP3*, markers for spermatocytes, or *PRM1*, *PRM2*, *TP1*, and *TP2*, hallmarks for spermatids, respectively (Figure 1c). Together, these data implicate that the cells isolated by STAPUT are spermatogonia, spermatocytes, and spermatids, respectively, in biochemical phenotype.

Immunofluorescence revealed that isolated human Sertoli cells from OA and SCO patients were positive for *GATA4*, *WT1*, and *VIMENTIN* (Figure 1d–1f). The purity of the isolated Sertoli cells was close to 100% as assessed by the above three marker expression. The isolated human spermatogonia were positive for *GFRA1* (Figure 1g, upper panel), and the isolated human spermatocytes were positive for *PIWIL2* (Figure 1g, middle panel). Meiosis spread assays reflected that the freshly isolated cells were co-expressed *SCP3*, *MLH1*, and *CREST* (Figure 2), markers for spermatocytes. In addition, the isolated human spermatids were positive for *ACROSIN* (Figure 1g, lower panel). No positive staining was observed in negative controls without primary antibodies (Figure 1h). The purity of isolated spermatogonia, spermatocytes, and spermatids was assessed to be ~90% according to their specific protein expression.

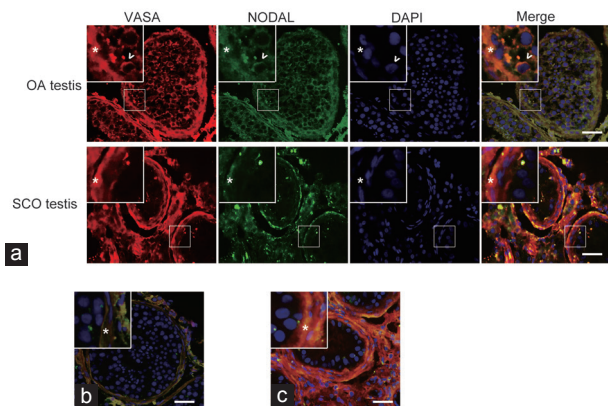
**Expression of NODAL and its multiple receptors in human testis**

To probe whether NODAL play a role in testicular development, we determined the expression of NODAL and its multiple receptors, including *ALK4*, *ALK5*, *ALK7*, *ACTR-IIB*, *CRIP1*, and *TGDF1*, in



**Figure 2:** Meiotic spread assays revealed the expression of *SCP3* (red fluorescence), *CREST* (blue fluorescence), and *MLH1* (green fluorescence) in the isolated spermatocytes. Scale bar = 10  $\mu$ m.

human testis and isolated Sertoli cells and male germ cells. To explore expression profiles of NODAL in testis tissue, *in situ* immunocytochemistry was performed. We found that NODAL was detected in VASA-positive germ cells in testes from OA patients, while it was hardly seen in human Sertoli cells or interstitial cells. No specific staining of NODAL protein was observed in SCO species (Figure 3). Moreover, NODAL's receptors ALK4, ALK7, and ACTR-IIB were detected in OA and SCO testis. Specially, ALK4, ALK7, and ACTR-IIB were expressed in Sertoli cells and most male germ cells in the seminiferous tubules of OA patients (Figure 4a and a'). In SCO patients, these receptors were detected in Sertoli cells (Figure 4b and b'). To verify the expressions profiles of NODAL signaling molecules, we performed RT-PCR showing that NODAL mRNA was not detected in isolated human Sertoli cells. In contrast, the transcripts of ALK4, ALK5, ALK7, ACTR-IIB, CRIPTIC, and TDGF1 were expressed in human Sertoli cells (Figure 5a). Additionally, ALK4, ALK7, and ACTR-IIB proteins were further detected in human Sertoli cells from both OA and SCO patients (Figure 5b–5e). Besides, the expressions of NODAL and its receptors in isolated germ cells were determined by RT-PCR and immunocytochemistry. RT-PCR showed that NODAL mRNA was expressed in isolated human spermatogonia and spermatocytes but not spermatids, while the transcripts of its receptors could be detected in all isolated germ cells (Figure 6a). Moreover, NODAL was found to be co-localized with VASA in isolated human germ cells by immunocytochemistry (Figure 7a). Furthermore, Double immunostaining, using NODAL and different makers for male germ cells, revealed that NODAL was expressed in isolated human spermatogonia and spermatocytes but not spermatids (Figure 7b and 7c). In addition, NODAL's receptors were detected in isolated human spermatogonia, spermatocytes, and spermatids (Figure 6b–6d). Together, these data suggest that NODAL is present in human male germ cells rather than in human Sertoli cells and that NODAL may act via a paracrine pathway in human Sertoli cells since multiple receptors for NODAL were detected in human Sertoli cells. In addition, NODAL may regulate germ cells via autocrine and/or paracrine pathways as its receptors exist on male germ cells.



**Figure 3:** Expression profiles of NODAL protein in OA and SCO testis. (a): Immunocytochemical staining showed that NODAL was expressed in VASA-positive cells in OA testis. No specific staining was seen in Sertoli cells and interstitium. There was no specific VASA and NODAL staining in SCO testis. There was nonspecific staining of the enclosed tubules and interstitium in both OA and SCO testis, which could also be seen in negative control slides (b and c). “\*” indicated the basement membrane, while “^” indicated the spermatogonia. (b and c): Negative control of immunocytochemistry of the human testes. Notes – OA: obstructive azoospermia, SCO: Sertoli cell-only syndrome. Scale bars = 50 μm.

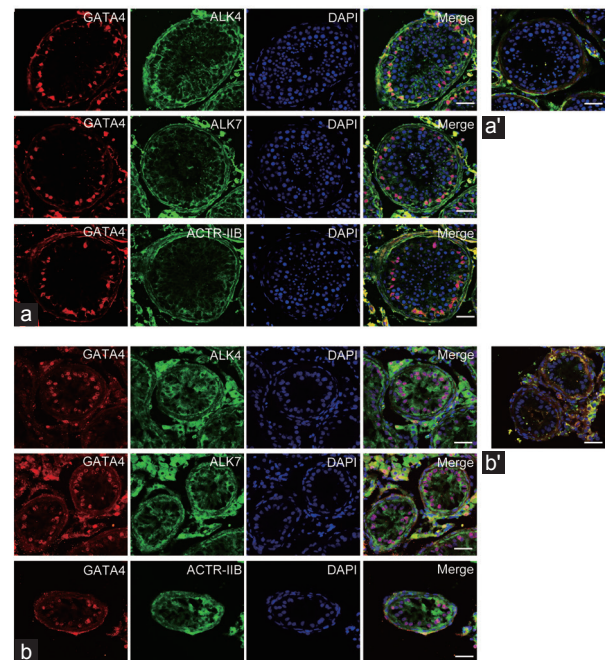
**NODAL promoted human Sertoli cell proliferation**

The effect of NODAL on human Sertoli cell proliferation was assessed by CCK-8 assay. Proliferation assays demonstrated that human recombinant NODAL enhanced the growth of human Sertoli cells of OA and SCO patients via dose- and time-dependent manners. Both OA and SCO Sertoli cells proliferated with high activity under NODAL treatment, and 100 ng ml<sup>-1</sup> of NODAL was the best concentration for promoting Sertoli cell proliferation after 24 h and 72 h of culture. Sertoli cells from OA and SCO patients assumed a highly proliferative activity till the fifth day of culture (Figure 8a). These results indicate that NODAL could stimulate the proliferation of human Sertoli cells.

To better understand the exact mechanism by which NODAL affects human Sertoli cell proliferation, we further examined expression changes of cell cycle regulators, including CYCLIN A, CYCLIN D1, and CYCLIN E. No remarkable change was found in the expression of these cell cycle proteins in human Sertoli cells without or with NODAL and SB431542 treatment (Figure 8b and 8c), suggesting that CYCLIN A, CYCLIN D1, and CYCLIN E were not involved in the proliferation of human Sertoli cells by NODAL.

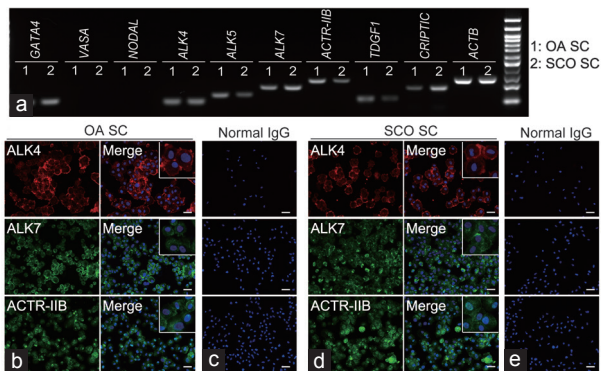
**NODAL enhanced the genes expressions of growth factors of human Sertoli cells**

Previous studies have demonstrated that Sertoli cells could secrete numerous growth factors, including GDNF, SCF, and BMP4, which regulates both SSC self-renewal and male germ cell differentiation. It has been proved that germ cells at different stages could produce various factors that regulate Sertoli cell function. We found that mRNA expression of GDNF, SCF, and BMP4 in human Sertoli cells was



**Figure 4:** Expression profiles of NODAL's receptors in OA and SCO testis. (a): Immunohistochemical staining showed that ALK4, ALK7, and ACTR-IIB were detected in GATA4-positive Sertoli cells in OA testis. (a'): Negative control of immunocytochemistry of the OA testes, displaying nonspecific staining. (b): Immunohistochemical staining displayed that ALK4, ALK7, and ACTR-IIB were expressed in GATA4-positive Sertoli cells in SCO testis. No specific staining was seen in other cells. (b'): Negative control of immunocytochemistry of the OA testes without primary antibody. Scale bars = 50 μm. OA: obstructive azoospermia; SCO: Sertoli cell-only syndrome.





**Figure 5:** Expressions of NODAL and its multiple receptors in human Sertoli cells. (a): RT-PCR showed that the expressions of transcripts of *NODAL* and its receptors *ALK4*, *ALK5*, *ALK7*, *ACTR-IIB*, *TDGF1*, and *CRYPTIC*, in the isolated human Sertoli cells from OA and SCO patient testes. Passage 2 Sertoli cells were used in this experiment. (b and c): Immunocytochemistry revealed that *ALK4* (red fluorescence), *ALK7* (green fluorescence), and *ACTR-IIB* (green fluorescence) were detected in human Sertoli cells isolated from OA patient testes. No specific staining (c) was observed when cells were incubated with normal IgG instead of primary antibodies. (d and e): Immunocytochemistry revealed that *ALK4* (red fluorescence), *ALK7* (green fluorescence), and *ACTR-IIB* (green fluorescence) were expressed in human Sertoli cells isolated from SCO patient testes. No specific staining (e) was seen when cells were incubated with normal IgG instead of primary antibodies. The enlarged images were showed in the up-right of the merged pictures. Scale bars = 20  $\mu$ m. OA: obstructive azoospermia; SCO: Sertoli cell-only syndrome; 1: OA Sertoli cells; 2: SCO Sertoli cells.

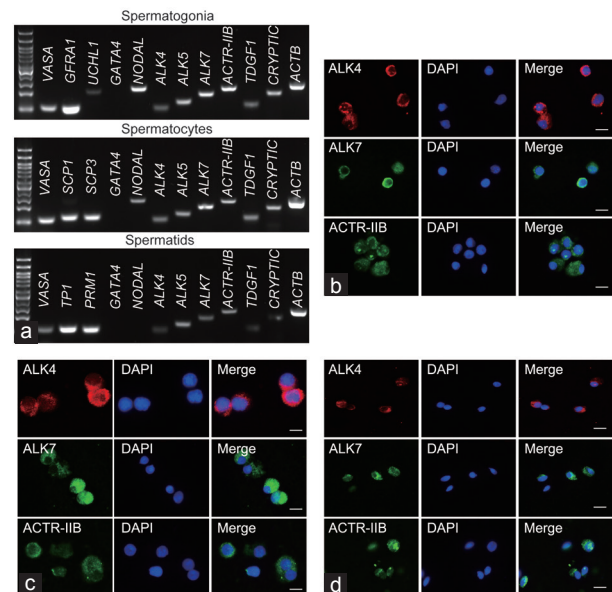
increased remarkably by NODAL treatment, whereas their transcripts were decreased by SB431542 treatment for 24 or 48 h (Figure 9a). It should be noted that there was not homogeneity of gene changes in Sertoli cells after NODAL and SB431542 treatment between OA and SCO patients. NODAL stimulated *GDNF* gene expression of Sertoli cells from both OA and SCO patients, whereas SB431542 reduced *GDNF* expression in OA patients, with no effect on *GDNF* transcription in SCO patients. NODAL increased *SCF* gene expression of Sertoli cells from OA patients, with no influence on *SCF* mRNA in SCO patients (Figure 9a).

Western blots were employed to detect protein expression of *GDNF*, *SCF*, and *BMP4*. NODAL exerted limited effect on the protein expression of *GDNF*, *SCF*, and *BMP4* of human Sertoli cells from both OA and SCO patients (Figure 9b and 9c). No obvious change of these proteins was found in human Sertoli cells after NODAL and SB431542 treatments, which was not in agreement with their gene regulation. These results indicated that NODAL could regulate gene expression of growth factors produced by Sertoli cells, including *GDNF*, *SCF*, and *BMP4*, and that there was a delay for affecting the translation of these proteins by NODAL.

## DISCUSSION

Several studies indicate that the members of TGF- $\beta$  superfamily exert biological effect on male germ cell and somatic cell differentiation, proliferation, and other function. In this study, we have demonstrated that NODAL, TGF- $\beta$  superfamily member, mainly produced by human spermatogonia and spermatocytes, could regulate the proliferation and expression of growth factors (e.g., *GDNF*, *SCF*, and *BMP4*) at transcriptional level of human Sertoli cells.

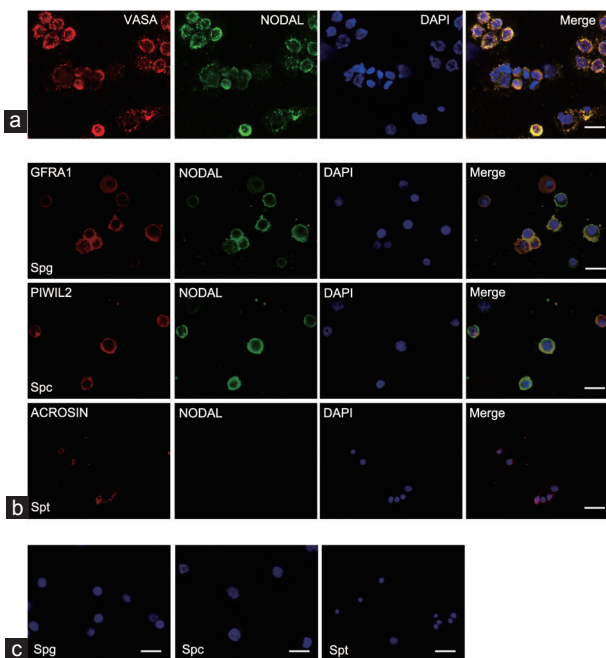
OA and SCO are common types of azoospermia in male infertility. OA patients are caused by the obstruction of genital tract duct and they have normal spermatogenesis, whereas SCO patients display the most severe impairment of spermatogenesis. The biopsies of SCO testes



**Figure 6:** Expressions of NODAL's multiple receptors in isolated human spermatogonia, spermatocytes, and spermatids. (a): RT-PCR showed that the expression of NODAL's multiple receptors *ALK4*, *ALK5*, *ALK7*, *ACTR-IIB*, *TDGF1*, and *CRYPTIC*, in the isolated human spermatogonia, spermatocytes, and spermatids from OA patient testes. (b): Immunocytochemistry revealed that *ALK4* (red fluorescence), *ALK7* (green fluorescence), and *ACTR-IIB* (green fluorescence) were expressed in human spermatogonia isolated from OA patient testes. (c): Immunocytochemistry revealed that *ALK4* (red fluorescence), *ALK7* (green fluorescence), and *ACTR-IIB* (green fluorescence) were detected in human spermatocytes isolated from OA patient testes. (d): Immunocytochemistry revealed that *ALK4* (red fluorescence), *ALK7* (green fluorescence), and *ACTR-IIB* (green fluorescence) were expressed in human spermatids isolated from OA patient testes. Scale bars = 20  $\mu$ m. OA: obstructive azoospermia; SCO: Sertoli cell-only syndrome.

in this study showed only Sertoli cells in seminiferous tubules with complete loss of male germ cells, while the histological characteristics of testis from OA patients were not completely identical to those of normal man testis. This was due to the obstruction of genital tract obstruction, which results in some alterations on the normal flow of seminiferous fluid and certain disruption of the seminiferous epithelium.

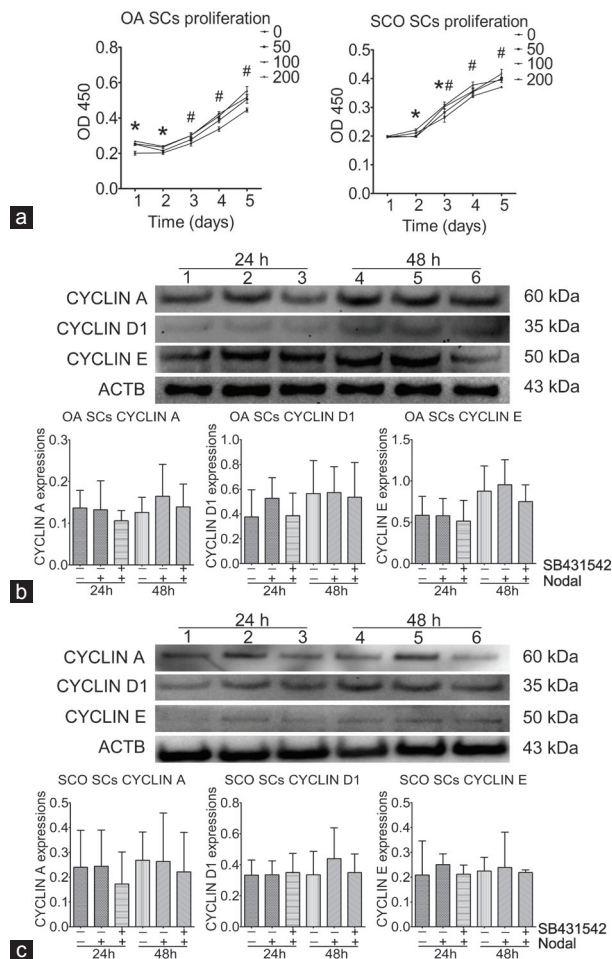
Using enzymatic digestion and differential plating and STAPUT method, we isolated human Sertoli cells, spermatogonia, spermatocytes, and spermatids from both OA and SCO patients. We have previously demonstrated that almost all the isolated human Sertoli cells are positive for both *GATA4* and *WT1*, specific markers for Sertoli cells.<sup>2</sup> In this study, we also detect the transcripts of numerous genes for Sertoli cells and other testicular cells, including *GATA4*, *WT1*, *ABP*, and *FSHR* in Sertoli cells, *HSD3B*, *CYP17A1*, *SMA*, *MYH11*, *CD34*, and *CD105* in Leydig cells, peritubular cells, and mesenchymal cells, respectively. RT-PCR and immunocytochemistry showed that the isolated human Sertoli cells were with high purity. STAPUT is an isolation method with less damage to cells.<sup>26,27,29</sup> It has been proved that spermatogonia, pachytene spermatocytes, and spermatids could be enriched from mouse and rat testes using this method. Thus, STAPUT strategy was employed in this study to isolate and enrich testicular male germ cell populations. *GFRA1*, *RET*, and *UCHL1* have been regarded as hallmarks for the presumptive spermatogonia,<sup>17,30</sup> while *SCP1*, *SCP3* and *PIWIL2* are specifically expressed in spermatocytes.<sup>31</sup> *PRM1*, *PRM2*, *TP1*, and *TP2* have been considered hallmarks for haploid spermatids.<sup>32</sup> RT-PCR analyses showed that the mRNA of these specific



**Figure 7:** Expression profiles of NODAL proteins in human germ cells. (a): Double staining showed that VASA (red fluorescence) and NODAL (green fluorescence) were co-expressed in human germ cells. (b): Immunohistochemistry revealed that NODAL (green fluorescence) was detected in spermatogonia (GFRA1: red fluorescence) and spermatocytes (PIWIL2: red fluorescence) but not in spermatids (ACROSIN: red fluorescence). (c): Negative control of immunocytochemistry of the human germ cells, which showed no specific staining. Scale bars = 20  $\mu$ m. Spg: spermatogonia; Spc: spermatocyte; Spt: spermatid.

genes was detected in spermatogonia, pachytene spermatocytes, and spermatids, respectively. Immunocytochemistry further confirmed that GFRA1, PIWIL2 and SCP3, and ACROSIN were expressed in more than 90% of the isolated spermatogonia, pachytene spermatocytes, and spermatids, respectively, reflecting a high purity of these isolated cells by STAPUT.

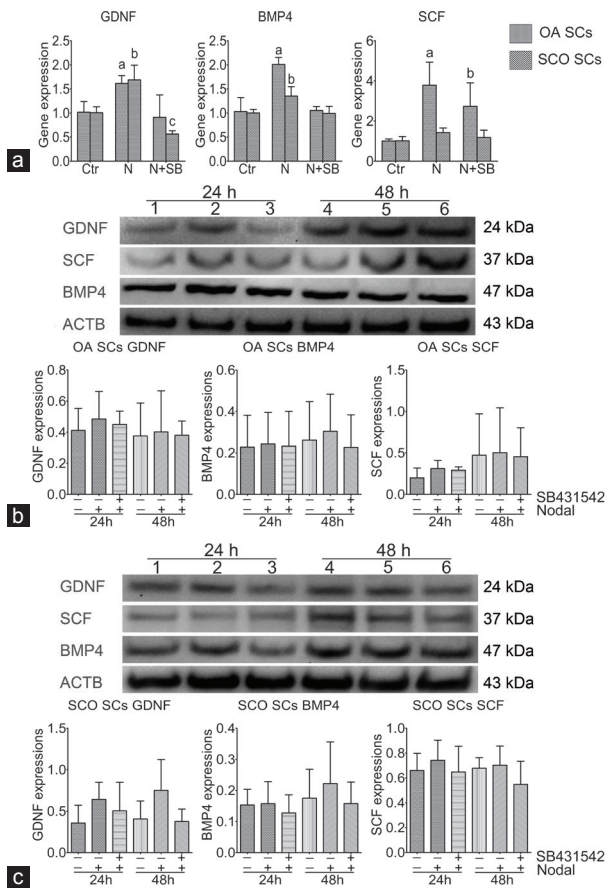
NODAL exerts its biological effect via interaction with their receptors in an autocrine or paracrine ways. NODAL is secreted from the cell and binds to its receptors ALK4, ALK7, and ACTR-IIB.<sup>14</sup> In addition, the epidermal growth factor-related protein cryptic family 1 (CRIPTIC) and teratocarcinoma-derived growth factor 1 (TDGF1) are considered to be essential to NODAL signaling.<sup>33</sup> In rodent studies, NODAL and its receptors, including ALK4, ALK7, ACTR-IIB, TDGF1 and CRIPTIC, could be identified in mouse fetal testis.<sup>34,35</sup> Additionally, NODAL and TDGF1 were found to be expressed in male gonad, with predominant expressions from 12.5 to 15.5 dpc periods. ALK4, ACTR-IIB, and TDGF1 were detected to be abundant in both purified germ and somatic cells in male gonads at 13.5 dpc.<sup>21</sup> ALK4 and ALK7 showed higher expression levels in somatic cells than those in germ cells in fetal testis.<sup>35</sup> NODAL's receptors ALK4, ALK7, and ACTR-IIB were demonstrated to be presented in germ cells and Sertoli cells at birth and in adulthood, while NODAL is only detected in germ cells, being absent in Sertoli cells.<sup>17,36</sup> In human studies, ACTR-IIB protein has been reported to be localized in Sertoli cells, gonocytes, and interstitial cells in the human fetal testis from week 13 to week 19 of gestation.<sup>37</sup> ALK4 and ACTR-IIB proteins could be detected in Sertoli cells, spermatogonia, and spermatocytes in both phenotypically normal and gonadotropin-deprived adult human testes.<sup>38</sup> Another study also



**Figure 8:** The influence of NODAL in human Sertoli cell proliferation and cell cycle proteins. (a): CCK-8 assay showed that NODAL promoted human Sertoli cell growth. OD values of Sertoli cells under 100 ng ml<sup>-1</sup> human recombinant NODAL treatment were remarkably higher than those of other groups from 24 h to 72 h of culture. “\*” indicates that 100 ng ml<sup>-1</sup> human recombinant NODAL treatment displayed the highest OD values when compared with those in other groups at the same time points ( $P < 0.05$ ). “#” indicates that OD values of each group at that time point was higher than that of the 24 h of culture. (b and c), Western blots reflected the effect of NODAL on the expression of CYCLIN A, CYCLIN D1, and CYCLIN E in human Sertoli cells from OA and SCO patients. Notes: In b and c, 1, 2, and 3 represented proteins changes of OA and SCO Sertoli cells with the control, NODAL treatment, and NODAL and SB431542 treatment, respectively, for 24 h; 4, 5, and 6 represented proteins changes of OA and SCO Sertoli cells with control, NODAL treatment, and NODAL and SB431542 treatment, respectively, for 48 h. OA: obstructive azoospermia; SCO: Sertoli cell-only syndrome.

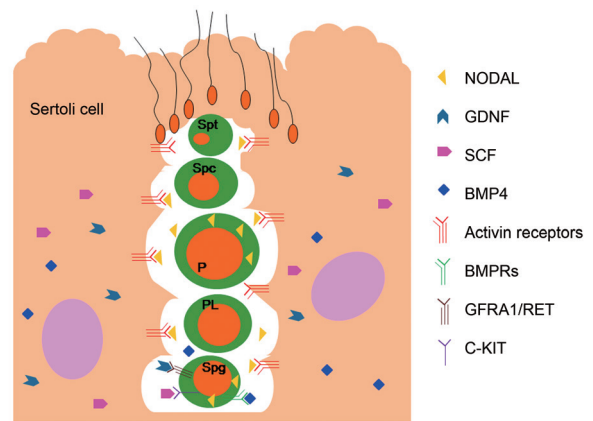
showed that ALK5 gene and protein were presented in normal testis and pathological testis from SCO and hypospermatogenesis patients.<sup>39</sup> Thus, it can be speculated that NODAL signaling play crucial roles in testis function. However, expression profiles of NODAL signaling molecules in different testicular cells in OA and SCO testes are still unknown. In this study, we found that NODAL transcript and protein were expressed in human spermatogonia and spermatocytes but not in spermatids or in Sertoli cells, while its receptors were presented in Sertoli cells, implicating that NODAL may act as a regulator of human Sertoli cells via a paracrine pathway. Our findings were partly consistent with previous study showing that NODAL was expressed in mouse spermatogonia but not in spermatocytes.<sup>17</sup> Meanwhile, it





**Figure 9:** The effect of NODAL on the expression of several growth factors at transcription and translation levels in human Sertoli cells. (a): Real-time PCR showed the expression of *GDNF*, *SCF*, and *BMP4* transcripts in OA Sertoli cells without or with NODAL and SB431542 treatment. “a” represented statistical difference compared with control group in OA Sertoli cell gene regulation. “b” represented statistical difference compared to control group in SCO Sertoli cell gene regulation ( $P < 0.05$ ). (b and c): Western blots displayed the protein expression of GDNF, SCF, and BMP4 in human Sertoli cells without or with NODAL and SB431542. Notes: In a, Ctr: control group; N: NODAL group; N + SB: NODAL and SB431542 group. In b and c, 1, 2, and 3 represented protein changes of OA and SCO Sertoli cells with control, NODAL, or NODAL and SB431542 treatment, respectively, for 24 h; 4, 5, and 6 represented protein changes of OA and SCO Sertoli cells with control, NODAL, and NODAL and SB431542 treatment, respectively, for 48 h. OA: obstructive azoospermia; SCO: Sertoli cell-only syndrome.

should be noted that ALK4, ALK7, and ACTR-IIB were also detected in human male germ cells, reflecting that NODAL play potential roles in mediating the fate decisions of human male germ cells. These results were consistent with previous studies in rodent studies. Additionally, it was actually demonstrated that NODAL could promote proliferation of mouse SSC.<sup>17</sup> Therefore, considering these findings, it could not be ruled out the possibility that NODAL could also regulate germ cell function in human testis through autocrine and/or paracrine pathway. Interestingly, some of the Sertoli cells from both OA and SCO testes showed nuclear staining of the receptors, which might be due to the fact that these receptors may shuttle from the cell surface to the nucleus. Consistently, this phenomenon has been reported for a number of growth factors and receptors.<sup>38,40</sup> NODAL has been previously found mainly to exist in stem cells, which can regulate cell self-renewal (proliferation and survival).<sup>14,17</sup> In this study, we found that NODAL was also expressed in human spermatogonia and



**Figure 10:** Schematic diagram illustrated the cross-talk or interaction among male germ cells and Sertoli cells via NODAL signaling in human seminiferous tubules. NODAL is found to be secreted by spermatogonia and spermatocytes, which can exert its biological effect on regulating the proliferation and secretion of growth factors (e.g., GDNF, SCF, and BMP4) of human Sertoli cells. Sertoli cells can also produce other growth factors which play key roles in regulating spermatogenesis. The functional and appropriate interaction among male germ cells and Sertoli cells maintains the normal spermatogenesis. Spg: spermatogonia; PL: preleptene; P: pachytene spermatocyte; Spc: spermatocyte; Spt: spermatid.

spermatocytes, suggesting that it may regulate cell mitosis and meiosis of human male germ cells. However, it remains to be defined for the specific roles of NODAL in regulating human spermatogenesis.

It is traditionally considered that Sertoli cells proliferate during fetal and early postnatal development. And Sertoli cell proliferation cease till puberty and maintain a certain level throughout the life. Nevertheless, this concept has been challenged, as Sertoli cells from adult mammalian testes can possess proliferative ability.<sup>2,41,42</sup> It remains unknown whether the isolated Sertoli cells resume proliferation *in vitro* under specific conditions or a subpopulation of Sertoli progenitor cells maintain the proliferative activity.<sup>41</sup> NODAL signaling has been widely studied in stem cells. In fact, all key components of NODAL signaling have been demonstrated to be enriched in mouse and human stem cells. The autocrine NODAL factor could bind to its membrane receptors to promote the cell proliferation and maintain the undifferentiated state of stem cells.<sup>14</sup> Moreover, the extensive expression of NODAL and its receptors has been found in various kinds of cancer cells, especially cancer stem cells, which has been shown to be crucial for cancer cell proliferation, survival, and progression.<sup>43</sup> With regard to reproduction, it has been reported that NODAL signaling is active in XY germ cells during mouse gonad development. Genetic suppression of NODAL signaling led to fewer proliferating germ cells compared with those in normal at 13.5 dpc. Additionally, high level expression of NODAL was found in testicular germ cell tumor, which indicated that its overexpression maintained the high proliferative activity and pluripotent state of germline stem cells and prevented germ cells from full differentiation.<sup>21</sup> Previous study showed that blockade of signaling through ALK4/7 in male gonad at E11.5 caused remarkable reduction of Sertoli cell proliferation.<sup>35</sup> In this study, proliferation assays showed that NODAL could stimulate the proliferation of human Sertoli cells. This result was in agreement with previous study showing that NODAL promoted mouse SSC proliferation.<sup>17</sup> In addition, the promoting effect was optimal at 100 ng ml<sup>-1</sup> of NODAL within 72 h of treatment, suggesting this effect is dose- and time-dependent. Cell cycle proteins play important roles in regulating cell mitosis. It has



been reported that CYCLIN A is a key protein regulating S phase of cell cycle, while CYCLIN D1 and CYCLIN E play a regulatory role during the G1/S-phase transition.<sup>17,30</sup> In our study, we detected CYCLIN proteins' expression to elucidate the possible mechanism underlying the proliferation of human Sertoli cells by NODAL. However, no remarkable changes of these cell cycle proteins were found after NODAL or SB431542 treatment, suggesting that NODAL does not act via the regulation of CYCLIN A, CYCLIN D1, and CYCLIN E to induce the proliferation of human Sertoli cells. In fact, there may be several other events involved in regulating cells proliferation.<sup>44,45</sup> Previous study demonstrated that NODAL could activate Smad2/3 and Oct-4 signaling, eventually resulting in proliferation of mouse SSC.<sup>17</sup> In our study, we demonstrated the stimulatory effects of NODAL on human Sertoli cells. Thus, we postulated that NODAL may affect human Sertoli cells proliferation through this pathway, which needs to be verified further.

Sertoli cells are able to secrete various kinds of factors, including GDNF, SCF, and BMP4, which play vital roles in regulating self-renewal and proliferation of SSCs during spermatogenesis.<sup>8,46,47</sup> Additionally, we have recently revealed that GDNF, SCF, and BMP4 in Sertoli cells from NOA patients were remarkably lower than those in Sertoli cells from OA patients.<sup>2</sup> However, the regulation of these growth factors is still unknown. There are cell-to-cell cross talk in the testis and other tissues, which are actually mainly performed by various growth factors and cytokines.<sup>48</sup> NODAL can interact with others in several biological process. Interactions between NODAL and BMP signaling pathways have been exhibited in various biological regulation.<sup>49,50</sup> In this study, we found that NODAL enhanced the expression of *GDNF*, *SCF*, and *BMP4* at transcriptional levels. Notably, both NODAL and SB432542 positively regulates *SCF* transcript expressions. It can be explained that certain genes regulation are actually complicated and associated with many factors. Cross-talk among several factors is involved in Sertoli cell biological functions. SCF belongs to SCF/C-KIT system, and regulations of SCF may be more complicated than those of others. There is not homogeneity of gene expression changes after treatment of NODAL and SB431542 in Sertoli cells from OA and SCO patients. As was shown above, *SCF* mRNA expressions of Sertoli cells from OA and SCO patients showed different response when the cells treated with NODAL. *GDNF* mRNA expression was also different when these two kinds of cells were treated with SB431542. SCO is a kind of severe spermatogenesis damage that seminiferous tubules are lined by Sertoli cells only, with complete depletion of male germ cells. It is considered that Sertoli cell maturation arrests and their dysfunctions are the key factors, leading to these spermatogenic disorders. Sertoli cell abnormalities may display the dysfunctions of cross-talk with other cells or unusual response to other growth factors,<sup>2,8,51</sup> which may account for the different response of OA and SCO Sertoli cells to NODAL stimulation. Furthermore, the results of Western blots was not coincident with that of quantified PCR, which may be owing to the complexity of protein expression and regulation, including posttranscriptional regulation. Besides, expressions of proteins did not always correlate with those of genes, which might be due to the fact that protein expression and their modulations are generally transient and temporary.<sup>17,46</sup> However, it should be noted that we have found that NODAL signaling did not affect expressions of GDNF, BMP4, and SCF proteins, but it remains possible that the secretion of another crucial growth factors or cytokines could be modulated or induced by NODAL signaling. Taken together, the findings from peers and us indicate that NODAL may be involved in regulation of human spermatogenesis including the proliferation and differentiation of male germ cells.

Researchers have been exploring a number of factors and cytokines participating in the maintenance of spermatogenesis microenvironment. As we summarized in **Figure 10**, NODAL, produced by human spermatogonia and spermatocytes, could stimulate the growth of human Sertoli cells and enhance the mRNA expressions of GDNF, BMP4, and SCF in human Sertoli cells. This study illustrates the regular cross-talk between male germ cells and human Sertoli cells for normal testicular function and spermatogenesis.

## AUTHOR CONTRIBUTIONS

RHT performed the experiments and data analysis and manuscript writing. SY, ZJZ, JLW, YL, and CY assisted with cell isolation and culture. MM, YG, QY and YH assisted with gene and protein detection. YRH, ZH and ZL designed the experimental strategy and revised the manuscript.

## COMPETING INTERESTS

All authors declare no competing interests.

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Supplementary information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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