

## C-type natriuretic peptide stimulates function of the murine Sertoli cells *via* activation of the NPR-B/cGMP/PKG signaling pathway

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**C-type natriuretic peptide (CNP) is an important regulator of the male reproductive process. Our previous investigations showed that CNP can significantly stimulate the mRNA expression of androgen-binding protein (Abp) and transferrin (Trf) in the rat Sertoli cells, but the pathways responsible for this process remain to be elucidated. We predict that CNP binds the natriuretic peptide receptor B (NPR-B) to regulate expression of ABP and TRF through the intracellular cyclic guanosine monophosphate (cGMP) pathway. To address this question, in this study, we first confirmed the expression and localization of CNP and NPR-B in rat testes by immunohistochemistry and western blotting. Then, ELISA and real-time PCR were performed to investigate the signaling pathway of CNP in Sertoli cells in rat testes. Our results showed that CNP was mainly localized in the germ cells and Leydig cells, and its receptor, NPR-B, was mostly expressed in the Sertoli cells and vascular endothelial cells. CNP supplementation in the Sertoli cell medium was accompanied by an increase in the amount of intracellular cGMP and in the production of Abp and Trf mRNA, whereas inhibition of PKG with KT5823 led to a decrease in the expression of Abp and Trf mRNA. Moreover, Abp and Trf mRNA were no longer elevated when we used liposome-mediated RNA interference technology to silence the NPR-B gene in a mouse Sertoli cell line (TM4). These results suggest that CNP contributes to the regulation of ABP and TRF in the Sertoli cells through the NPR-B/cGMP/PKG signaling pathways.**

**Keywords:** C-type natriuretic peptide; natriuretic peptide receptor-B; Sertoli cell; cyclic GMP

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**Abbreviations:** Abp, androgen-binding protein; Trf, transferrin; *Npr-b*, natriuretic peptide receptor-B

### INTRODUCTION

The natriuretic peptide family in mammals mainly consists of four structurally related peptides: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP) and D-type natriuretic peptide (DNP) (Moghtadaei *et al.*, 2016). As the third member of the natriuretic peptide family, the C-type natriuretic peptide (CNP) was initially purified in 1990 from por-

cine brain extracts for its ability to relax smooth muscle (Sudoh *et al.*, 1990; Xia *et al.*, 2013). In contrast to ANP and BNP, which are both cardiac hormones (Baba *et al.*, 2019), CNP is regarded as an endothelin and plays its role through specifically binding the membrane-bound natriuretic peptide receptor B (NPR-B) via intracellular cyclic guanosine monophosphate (cGMP) activation (An *et al.*, 2019). The literature has shown that CNP is involved in mediating a variety of physiological effects, such as the regulation of myocardial contractility (Prickett *et al.*, 2017), cell proliferation (Zenitani *et al.*, 2016), endochondral ossification (Bükülmez *et al.*, 2014), and reproduction (Zhang *et al.*, 2010). Sogawa and others (Sogawa *et al.*, 2014) created an NPR-B deficient male mouse model with short limbs and dwarfism, which turned out to be infertile, with pathological sections that indicated a puberty spermatogenesis delay, but its mechanism was unclear.

The Sertoli cells are somatic cells in seminiferous tubules that play an extremely important role in maintaining a subtle testicular microenvironment. They are the main element of the blood-testis barrier (Smith & Braun, 2012), and synthesize and secrete androgen-binding protein (ABP), transferrin (TRF) and other important functional proteins to regulate the process of spermatogenesis (Ma *et al.*, 2015). ABP is a glycoprotein produced by the Sertoli cells that specifically binds to testosterone, which enables spermatogenesis in the seminiferous tubules and sperm maturation in the epididymis (Chinta *et al.*, 2015). TRF, secreted by the Sertoli cells, is an important marker of its function, providing essential iron for spermatogenic cells (Dietrich *et al.*, 2010). Xia *et al.* (2007) reported that CNP could perturb the Sertoli cell tight junctions, causing the disappearance of the blood-testis barrier-associated proteins (JAM-A, occludin, N-cadherin and  $\beta$ -catenin) from the cell/cell interface. Our previous investigation showed that CNP could significantly stimulate in vitro cultured Sertoli cells to express Abp, inhibin B (Inh-b) and Trf, which play a key role in spermatogenesis (Huang *et al.*, 2011).

This study was designed to elucidate the signal transduction of CNP in Sertoli cells. Furthermore, there is no literature about the regulation of the endocrine function of Sertoli cells through cGMP signal transduction.

### MATERIALS AND METHODS

#### Animals

Male Sprague-Dawley rats were obtained by the Laboratory Animal Centre of Tongji Medical College, Huazhong University of Science and Technology (Wu-

han, China). They were raised under controlled environmental conditions ( $22 \pm 2^\circ\text{C}$ ; 12 h light/12 h dark cycle; 35–60% relative humidity) with food and water ad libitum. This study was approved by the Centre of Experimental Animals of Huazhong University of Science and Technology (no. 4200980001411, 10-10-2015) and conducted in the reproductive immunology laboratory at the Reproductive Health Institute of Tongji Medical College.

#### Immunohistochemical detection of CNP and NPR-B in rat testes

Testes collected from adult male Sprague-Dawley rats were used to analyze the localization of CNP/NPR-B by an immunohistochemical staining method. Briefly, the testicular tissue was fixed in the Bouin's acid for 24 h and then processed for routine paraffin embedding. Paraffin sections were dewaxed in xylene and rehydrated through descending concentrations of ethanol, and then endogenous peroxidases were blocked with 3%  $\text{H}_2\text{O}_2$ /methanol. The sections were preincubated with non-immune rabbit serum to minimize nonspecific staining. After that, the testicular tissue was incubated with primary rabbit polyclonal antibody against CNP (sc-20952, 1:50) and goat polyclonal antibody against NPR-B (sc-16870, 1:50) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and then incubated with a biotinylated secondary antibody (rabbit anti-goat IgG 1:200, Boster Biotechnology Co., Ltd., probed, Wuhan, China) and the streptavidin-biotin-peroxidase complex (Boster Biotechnology Co. Ltd., Wuhan, China) according to the manufacturer's instructions. Peroxidases were observed with a diaminobenzidine reagent, and the sections were briefly counterstained with hematoxylin. Negative controls were prepared without the primary antibody. The experiment was repeated three times.

#### Isolation of the Sertoli cells and Leydig cells

Primary rat Sertoli cells were isolated and cultured as previously described (Huang *et al.*, 2007). Briefly, testes from 18- to 22-day-old SD rats were removed, decapsulated, chopped and sequentially digested with three enzymes at  $37^\circ\text{C}$ : first with 0.25% trypsin for 15 minutes, then with 0.1% hyaluronidase for 30 minutes, and finally with 0.1% collagenase V for 2–3 hours. The isolated cells were incubated at  $32^\circ\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$ . To increase the purity of the Sertoli cells, the cultured cells were subjected to a hypotonic shock (treatment) with 20 mmol Tris-HCl after 48 hours of incubation. The cultured cells were identified by Feulgen staining and in situ hybridization with digoxin-labeled rat ABP cDNA. More than 95% of the cultured cells were Sertoli cells.

Primary rat Leydig cells were isolated and cultured as follows (Zhou *et al.*, 2016). Briefly, testes from adult SD rats were removed and decapsulated, and then placed in DMEM-F12 containing 0.25 mg/mL collagenase with constant agitation. The supernatant containing Leydig cells was filtered through a double layer of 100  $\mu\text{m}$  nylon mesh and collected by centrifugation. The Leydig cell suspension was loaded on top of a discontinuous Percoll gradient (5%, 30%, 58% and 70%) and then centrifuged. After centrifugation, most of the purified Leydig cells were observed in the third Percoll gradient. These Leydig cells were carefully collected and washed two times. The isolated cells were incubated at  $37^\circ\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$ . The cultured cells were identified by  $3\beta$ -hydroxysteroid dehydrogenase ( $3\beta$ -HSD)

staining. Approximately 90% of the cultured cells were Leydig cells.

#### Protein extraction and western blot analysis

The total proteins of testes, cultured Sertoli cells and Leydig cells were extracted using a radioimmunoprecipitation assay lysis buffer according to the manufacturer's recommendation. Concentrations of proteins were estimated by an enhanced BCA protein assay kit (P0010). Equal amounts of proteins were separated on 10% SDS-PAGE gels and transferred onto polyvinylidene fluoride membranes. The immunodetection of NPR-B was performed with the use of NPR-B goat polyclonal antibody (diluted at 1:200) and a secondary antibody conjugated with HRP (diluted at 1:5000). The reactive bands were detected by an enhanced chemiluminescence system using X-ray film. The immunoreactive bands were analyzed as two-dimensional images using ImageJ (version 1.32). The results are expressed as the mean  $\pm$  S.D. from three independent experiments.

#### Measurement of intracellular cGMP levels

Before experiments, the cells were pretreated overnight in a serum-free medium. Subsequently, the Sertoli cells in the experimental groups were incubated with different concentrations ( $10^{-7}$  mol/L,  $10^{-6}$  mol/L) of CNP (N8768, Sigma-Aldrich, St. Louis, MO, USA), and the control group was incubated with an untreated medium. After 2 h treatment, cGMP was extracted from adherent cells by treatment with 0.1 M HCl. Samples were stored at  $-80^\circ\text{C}$  before measurement of cGMP levels. cGMP concentrations were measured by a rat cGMP ELISA kit (JL11179, Jianglai Biology, Shanghai, China,) according to the manufacturer's protocol. The results are expressed as the mean  $\pm$  S.D. from three independent experiments.

#### RNA isolation and RT-qPCR

Purified Sertoli cells were incubated in the presence of CNP, 8-Br-cGMP (cell-permeable cGMP analog 8-bromoguanosine 3',5'-cyclic monophosphate), CNP + KT5823 (PKG inhibitor) or PBS (control) at the same time for 2 h, and then the treated cells were harvested to analyze gene expression levels of Abp and Trf using RT-qPCR. Total RNA from purified Sertoli cells was isolated using TRIzol reagent (Life Technologies, Gaithersburg, MD) following the protocol recommended by the manufacturer. Total RNA concentration and purity were determined spectrophotometrically. To eliminate residual genomic DNA, RNA samples (3  $\mu\text{g}$  of total RNA) were treated with 1 IU of DNase-I in a 10- $\mu\text{l}$  reaction mixture. After DNase-I treatment, 3  $\mu\text{g}$  of total RNA from each sample were reverse transcribed into first-strand cDNA in a 20- $\mu\text{l}$  reaction mixture containing 0.5  $\mu\text{g}$  of oligo(dT)<sub>18</sub> primer, 1 $\times$ RT buffer, 1 mM dNTP Mix, 20 U of RiboLock RNase Inhibitor and 200 U of RevertAid M-MuLV Reverse Transcriptase according to the supplier's instructions.

cDNA samples were subjected to real-time qPCR (Roche LightCycler 480 Real-Time PCR), which was performed as previously described (Huang *et al.*, 2011). The primer pairs used in RT-PCR are listed in Table 1. An aliquot of 5  $\mu\text{l}$  of the RT reaction was amplified with the PCR reagent system in a final volume of 50  $\mu\text{l}$  containing 1 $\times$ Taq Buffer, 3 mM  $\text{MgCl}_2$ , 0.2 mM dNTP Mix, 0.2  $\mu\text{M}$  primers (Table 1), 1.25 U of Taq DNA polymerase and 0.2 $\times$ SYBR Green I. The amplified products were analyzed by 2% agarose gel electrophoresis and vis-

**Table 1. Sequences of primers used in the RT-PCR analysis**

Gene	Forward primer	Reverse primer	Length (bp)
<i>β-actin</i>	5' TCC TCC CTG GAG AAG AGC TA 3'	5' TCA GGA GGA GCA ATG ATC TTG 3'	302
<i>r-Abp</i>	5' ATC AGC AAA CCC TCT TCC CTC 3'	5' AGG CAT AGC ATC TCT CTT TTC C 3'	263
<i>r-Trf</i>	5' CCA AGC TCC AAA CCA TGT TGT 3'	5' GCA GGC TTC TAG GAG TCG TGA 3'	283
<i>Npr-b</i>	5' CCAAATTCTACCCCACTCTG 3'	5' TAAGAAGTGTACCACCTGC 3'	183

**Abbreviations:** Abp, androgen-binding protein; Trf, transferrin; *Npr-b*, natriuretic peptide receptor-B.

ualized with ethidium bromide. The results are expressed as the mean  $\pm$  S.D. from three independent experiments.

#### TM4 cell transfection

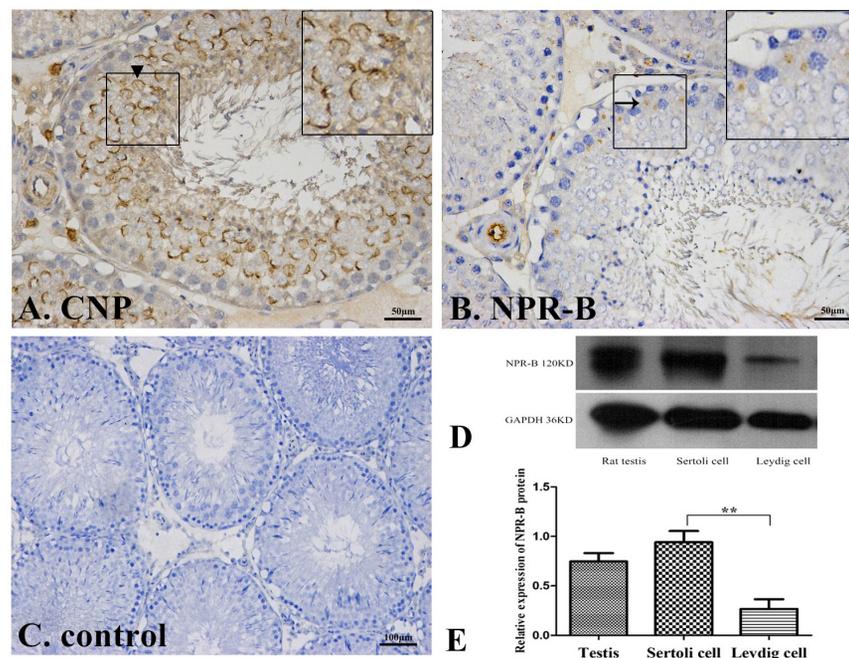
Due to the low transfection efficiency of the rat primary Sertoli cells, we chose the mouse Sertoli cell line TM4 (CRL-1715) for the transfection experiment. The mouse Sertoli cell line TM4 was purchased from ATCC. The cells were inoculated with approximately  $1 \times 10^5$  cells per well in 24-well plates, and 0.5 ml of medium per well was added. The next day, when the cells reached 70–90% confluence, the transfection experiment was completed following Invitrogen's Lipo-2000 protocol. Briefly, 5  $\mu$ l of Lipo-2000 reagent was diluted in 50  $\mu$ l of Opti-MEM, 5  $\mu$ g of DNA was diluted in 50  $\mu$ l of Opti-MEM, and the diluted DNA and Lipo-2000 reagent (1:1 ratio) were combined. The mix was incubated for 5 minutes at room temperature, then the DNA-lipid complex was added to the cells for an incubation period of 48 h at 37°C. Four shRNA interference sequences purchased from the OriGene company (Catalog No: TG712192) were chosen and designed to screen for the most effective shRNA to silence the NPR-B gene. The cultured cells without shRNA were regarded as the control. The four sequences of shRNA are listed in Table 2.

**Table 2. Sequences of shRNA used in transfection experiments**

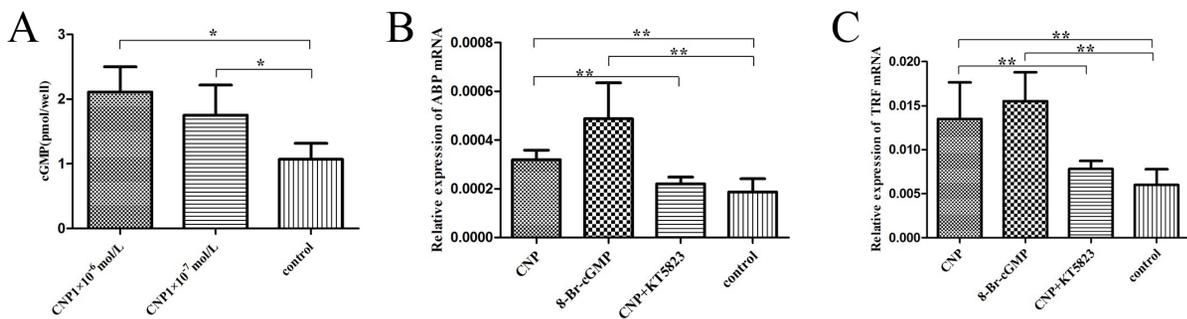
Gene name	Sequence
GI748769	GCTGTGGACTGCTCCAGAACTTCTTAGTG
GI748770	ATCAACTGCTGAACCAGATGACAGTCATG
GI748771	GCCTTTGACAGTGTCACTATCTACTTCAG
GI748772	CAACCTCTTGCTTCGCATGGAACAGTATG

#### Statistical analysis

All data were statistically analyzed using SPSS 23.0 software/Stata 21.0 software. The data shown are the mean  $\pm$  S.E.M. of at least three independent experiments. Each of the three to five independent experiments was run on a culture plate of Sertoli cells cultured in four to six replicates. Asterisks indicate significant differences ( $P < 0.05$ ) among means, as estimated by the Mann-Whitney unpaired nonparametric two-tailed test (for two-point data experiments), Kruskal-Wallis test followed by Dunn's multiple comparison test (for group comparisons) or one-way ANOVA followed by the Student-Newman-Keuls multiple-range test (for group comparisons).

**Figure 1. Expression of CNP and NPR-B in the normal rat testes.**

(A–C) Immunostaining of CNP/NPR-B in the rat testes. (A) Immunostaining of CNP in rat testes showed that CNP was mainly localized in the germ cells (mostly spermatocytes) in the seminiferous epithelium and in the Leydig cells in the interstitium. (B) Immunostaining of NPR-B in the rat testes showed that NPR-B was mostly distributed in the Sertoli cells and vascular endothelial cells. (C) Negative control staining in the rat testes (primary antibody omitted). (D–E) The expression of NPR-B in different kinds of cells in the testes. (D) Western blot of NPR-B. (E) Relative expression level of NPR-B according to D. The Sertoli cells were shown to express more NPR-B than the Leydig cells ( $P < 0.01$ ). NB: \*\* $P < 0.01$ , triangle: spermatocyte, arrow: Sertoli cell. NB: \* $P < 0.05$ , \*\* $P < 0.01$ .



**Figure 2. Dependence of CNP-induced ABP and TRF mRNA on the cGMP/PKG signaling pathway in Sertoli cells.**

(A) Effect of CNP on cGMP accumulation in the Sertoli cells. (B–C) Relative expression of ABP and TRF mRNA in Sertoli cells in different groups. CNP groups:  $1 \times 10^{-6}$  mol/L CNP; 8-bromo-cGMP groups:  $1 \times 10^{-4}$  mol/L 8-bromo-cGMP; CNP + KT5823 groups:  $1 \times 10^{-6}$  mol/L CNP +  $1 \times 10^{-6}$  mol/L KT5823; control groups: culture medium.

## RESULTS

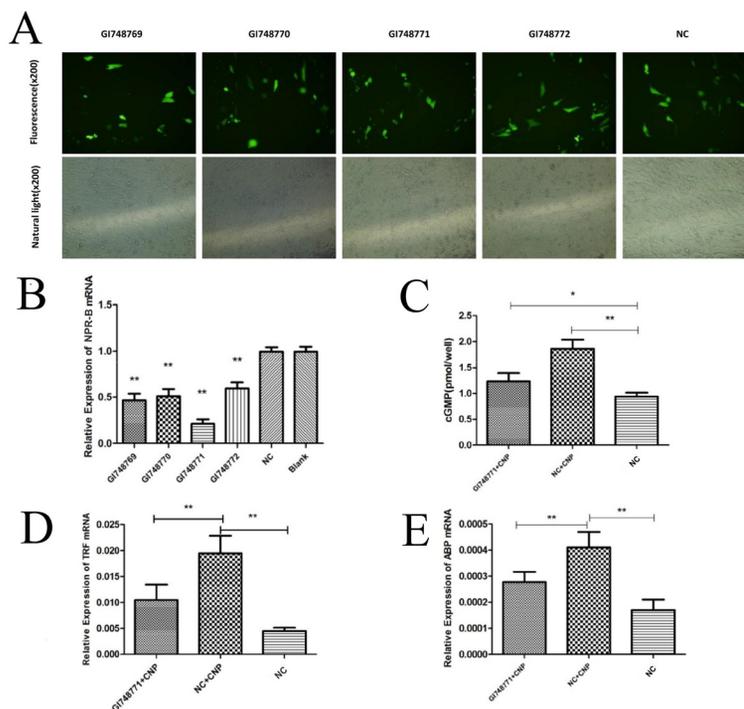
### Expression of CNP and NPR-B in rat testes

The immunohistochemical localization of CNP and NPR-B in rat testes is shown in Fig. 1A and B. Positive CNP staining was mainly localized in the germ cells (mostly spermatocytes) in the seminiferous epithelium and Leydig cells in the interstitium (Fig. 1A). However, its receptor, NPR-B, was mostly distributed in the Sertoli cells and vascular endothelial cells (Fig. 1B). The western blotting results further revealed that Sertoli cells and Leydig cells both expressed NPR-B (Fig. 1D), and Sertoli cells were shown to express more NPR-B than Leydig cells ( $P < 0.01$ , Fig. 1E), suggesting that the Sertoli cells are the major cell type involved in the expression of NPR-B protein in the seminiferous epithelium.

### cGMP/PKG signaling pathway participates in the CNP-induced expression of ABP and TRF mRNA in Sertoli cells

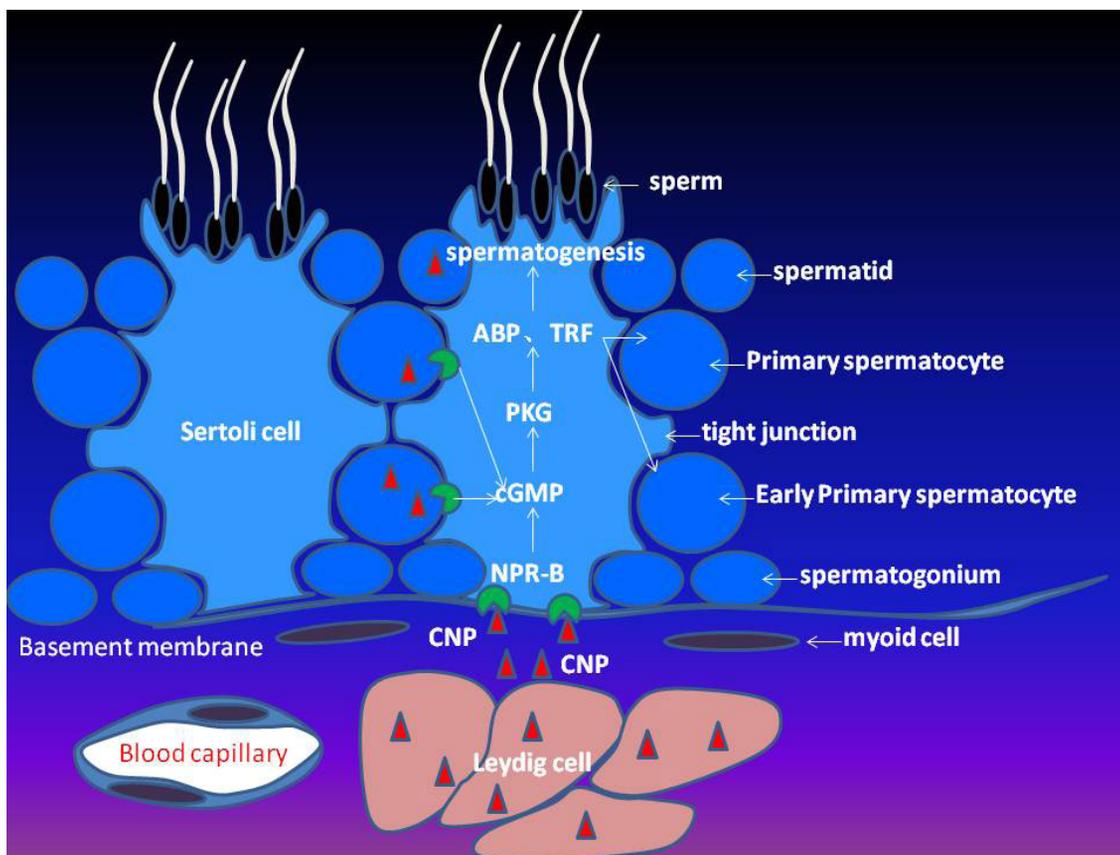
To investigate the effects of CNP on intracellular cGMP in Sertoli cells, cGMP levels in cultured Sertoli cells were measured using a cGMP ELISA kit after incubation with increasing concentrations of CNP. As shown in Fig. 2A, when we treated cells with  $1 \times 10^{-7}$  mol/L CNP and  $1 \times 10^{-6}$  mol/L CNP, the cGMP levels of both groups were significantly higher than in the control group ( $P < 0.01$ ). This result revealed that CNP could increase cGMP levels in Sertoli cells.

When compared with the control condition, supplementation with  $1 \times 10^{-6}$  mol/L CNP and  $1 \times 10^{-4}$  mol/L 8-bromo-cGMP in the culture medium has significantly increased gene expression of ABP and TRF in the cultured Sertoli cells, and this difference was statistically significant ( $P < 0.01$ , Fig. 2B and 2C). This result is consistent with our previous research (Huang *et al.*,



**Figure 3. CNP induces ABP and TRF mRNA expression through NPR-B as evidenced by using shRNA silencing in TM4 cells.**

(A) Transfection efficiency images of TM4 cells transfected with four different NPR-B shRNAs and the negative control group (NC). (B) Expression of NPR-B mRNA in transfected TM4 cells. (C) cGMP levels in cultured TM4 cells after 24 h incubation with  $1 \times 10^{-6}$  mol/L CNP. (D–E) Expression of ABP and TRF mRNA in cultured TM4 cells after 24 h of incubation with  $1 \times 10^{-6}$  mol/L CNP. NB: \* $P < 0.05$ , \*\* $P < 0.01$ .



**Figure 4. The signaling pathway of CNP in Sertoli cells.**

CNP secreted by germ cells and Leydig cells can bind NPR-B in Sertoli cells, increase ABP and TRF gene expression through cGMP/PKG signaling, and regulate spermatogenesis. CNP: C-type natriuretic peptide; NPR-B: natriuretic peptide receptor B; ABP: androgen-binding protein; TRF: transferrin; cGMP: cyclic guanosine monophosphate; PKG: cGMP-dependent protein kinase.

2011). Furthermore, KT5823 markedly inhibited the CNP-induced increase in the ABP and TRF mRNA expression in Sertoli cells ( $P < 0.01$ , Fig. 2B and 2C).

#### **Silencing the NPR-B gene attenuates the effect of CNP-induced expression of Abp and Trf mRNA in Sertoli cells**

To further clarify that CNP binds NPR-B to induce the expression of Abp and Trf mRNA, four different shRNAs were designed to transfect TM4 cells. The expression levels of NPR-B mRNA in TM4-transfected cells were analyzed by RT-PCR. The transfection efficiency images shown in Fig. 3A demonstrate that the transfection efficiency of the four shRNAs exceeded 50%. When compared with the negative control (blank plasmid), all four Npr-b shRNAs could inhibit expression of Npr-b mRNA, especially GI748771 ( $P < 0.01$ , Fig. 3B). Accordingly, we chose GI748771 to transfect TM4 cells for the 48 hour follow-up experiment.

The cGMP levels in cultured TM4 cells were measured after 24 h of incubation with  $1 \times 10^{-6}$  mol/L CNP. The amount of cGMP was significantly lower in the Npr-b silenced group than in the negative control group ( $P < 0.01$ ); moreover, the cGMP content in the Npr-b silenced group was higher than that in the blank group ( $P < 0.05$ , Fig. 3C), which is probably due to the 50% transfection efficiency of Npr-b shRNA (Fig. 3C). This is also shown in the expression of Abp and Trf mRNA ( $P < 0.01$ , Fig. 3D and 3E).

#### **DISCUSSION**

The effects of CNP on reproductive function have recently drawn increased attention (Mirczuk *et al.*, 2019). Recently, CNP was shown to play a critical role in reproduction (Guo *et al.*, 2020; Wu *et al.*, 2019), especially in the female reproduction (Zhang *et al.*, 2010). However, the effects of CNP on male reproduction have only been reported in a few studies, although there are high expression levels of CNP in male reproductive organs (Nielsen *et al.*, 2008; Chrisman *et al.*, 1993). In our previous study, we found that CNP could significantly stimulate the endocrine function of the rat Sertoli cells, but its mechanism was unclear (Huang *et al.*, 2011). This study demonstrated that CNP regulated the function of Sertoli cells via activation of the NPR-B/cGMP/PKG signaling pathway. To date, there have been no reports in the literature regarding the signaling pathway of CNP in testes. This study is the first to report that CNP plays its role through the NPR-B/cGMP/PKG signaling pathway in testes.

In the testis, O. Collin *et al.* reported that ANP was localized in spermatocytes and round spermatids within seminiferous tubules, while BNP and CNP were localized in the rat Leydig cells (Collin *et al.*, 1997). A previous report has shown that natriuretic peptides could stimulate testosterone production *in vivo*, with significant effects at concentrations  $\geq 1 \times 10^{-8}$  mol/l of ANP,  $\geq 1 \times 10^{-9}$  mol/L of BNP and  $\geq 1 \times 10^{-6}$  mol/L of CNP (El-Gehani *et al.*, 2001). Knockdown of NPR2 by RNAi

resulted in S phase cell cycle arrest, cell apoptosis, and decreased testosterone secretion in the mouse Leydig cells (Yang *et al.*, 2019). Xia and others (Xia *et al.*, 2007) found that CNP was also expressed in a stage-specific manner and localized predominantly at the blood-testis barrier in the seminiferous epithelium at stage VIII of the epithelial cycle; however, its receptor, NPR-B, is distributed almost exclusively in the Sertoli cells. In our study, immunohistochemistry and western blotting methods were performed to determine the localization of CNP and NPR-B in rat testes. The results showed that CNP is mainly expressed in germ cells and Leydig cells but that its receptor, NPR-B, is mostly localized in the Sertoli cells and vascular endothelial cells. Indeed, our results are consistent with those reported in the previous literature. It is suggested that Sertoli cells may be the major cell type involved in the CNP/NPR-B signaling in the seminiferous epithelium.

According to previous studies, CNP can not only perturb the Sertoli cell tight junctions (Xia *et al.*, 2007), but can also cause the Sertoli cells cultured *in vitro* to express Abp and Trf mRNA (Huang *et al.*, 2011). In this experiment, shRNAs were used to silence Npr-b in a rat Sertoli cell line (TM4), and the results showed that Npr-b shRNAs could attenuate the effect of CNP-induced expression of Abp and Trf mRNA in Sertoli cells. Thus, we presumed that CNP promoted expression of Abp and Trf by binding NPR-B in Sertoli cells. Then, the cGMP signal was investigated in the Sertoli cells in the next experiment.

cGMP is an important intracellular second messenger. It promotes substrate protein phosphorylation to mediate a variety of biological effects mainly through the activation of its downstream signaling molecule, cGMP-dependent protein kinase (PKG) (Tolone *et al.*, 2019). Previous research demonstrated that cGMP was widely involved in the regulation of male reproductive functions, such as the development of germ cells (Sillaste *et al.*, 2017), testosterone synthesis in the Leydig cells (Sokanovic *et al.*, 2013), erection of the penis (Boydens *et al.*, 2017), activation of sperm motility (Miraglia *et al.*, 2011) and sperm capacitation (Cisneros-Mejorado *et al.*, 2012). The early literature reported that PKG was expressed in the seminiferous epithelium of basal cells and was mainly localized in the Sertoli cells (Huang *et al.*, 2007). Lee *et al.* found that nitric oxide synthase (NOS) is an important physiological regulator of the Sertoli cell tight junctions via the sGC/cGMP/PKG pathway (Lee *et al.*, 2003). Li and others (Li *et al.*, 2017) found that carbon monoxide (CO) can activate the sGC signaling pathways to protect mouse Sertoli cells from hyperthermia-induced apoptosis. In this work, CNP increased the level of cGMP in Sertoli cells in a dose-dependent manner. 8-Bromo-cGMP exerts an obvious enhancing effect on the expression of Abp and Trf mRNA in Sertoli cells. The results indicated that CNP modulates the endocrine function of Sertoli cells by increasing intracellular cGMP levels. Furthermore, KT-5823, a specific inhibitor of PKG, was shown to remarkably inhibit the stimulatory effect of CNP on the expression of Abp and Trf mRNA in Sertoli cells, which indicated that CNP promotes expression of Abp and Trf via the cGMP/PKG pathway. In summary, we conclude that CNP is mainly synthesized and secreted by the Leydig cells and spermatogenic cells, and then binds to the specific receptor NPR-B in the cell membrane of Sertoli cells. It increases intracellular second messenger cGMP concentration and then activates the downstream signaling molecules' PKG activity to promote expression of Abp and Trf mRNA in the Sertoli cells to regulate

spermatogenesis (Fig. 4). However, when performing immunohistochemical staining, a proper positive control was not included, and thus further studies are needed to include a positive control for immunohistochemistry to confirm our findings.

In summary, our study verified that CNP regulated expression of Abp and Trf mRNA in the murine Sertoli cells through the NPR-B/cGMP signaling. Our study was the first to investigate the role of cGMP signaling in the endocrine function of Sertoli cells. Next, we aim to further study the role of CNP in spermatogenesis.

## Declarations of interest

None.

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