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The effects of neuromedin S on the hypothalamic-pituitary-testicular axis in male pigs *in vitro*

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ABSTRACT

Evidence has shown that neuromedin S (NMS) and its receptor (NMU2R) are expressed in the hypothalamus, pituitary, and testis of pigs. To determine the potential mechanisms of NMS, we systematically investigated the direct effects of NMS on the hypothalamic-pituitary-testicular (HPT) axis of male pigs *in vitro*. We initially confirmed that NMU2R distributed in isolated hypothalamic cells, anterior pituitary cells and Leydig cells using immunocytochemistry. Subsequently we investigated the direct effects of NMS on hormone secretion from cells (anterior pituitary cells and Leydig cells) treated with different doses of NMS. The results showed that NMS increase the release of LH and FSH from anterior pituitary cells and testosterone from Leydig cells. NMS up-regulated the expression of *NMU2R* and *GnRH* mRNAs in hypothalamic cells, *NMU2R*, *LH* and *FSH* mRNAs in anterior pituitary cells, and *NMU2R*, *STAR*, *P450* and *3β-HSD* mRNAs and the expression of PCNA and Cyclin B1 protein in Leydig cells; moreover, it down-regulated the expression of *GnIH* mRNA in hypothalamic cells. Using immunofluorescence staining and confocal microscopy, we also demonstrated the colocalization of NMU2R and AR or GnIH in Leydig cells. These data *in vitro* indicated that NMS may regulate the release and/or synthesis of LH, FSH and testosterone at different levels of the reproductive axis through NMU2R, which provided novel evidence of the potential roles of NMS in regulation of pig reproduction.

1. Introduction

In 2005, a neuropeptide called neuromedin S (NMS) that contains 36 amino acids was reported to have many physiological functions in mammals. This novel peptide was designated NMS because of its specific expression in the suprachiasmatic nucleus (SCN) (Mori et al., 2005). NMS was identified as an endogenous ligand for two orphan G-protein-coupled receptors, FM-3/GPR66 (NMU1R) and FM-4/TGR-1 (NMU2R), using a reverse-pharmacological technique (Mori et al., 2005). NMU1R and NMU2R had previously been identified as neuromedin U receptor type-1 and type-2, respectively (Fujii et al., 2000; Howard et al., 2000; Kojima et al., 2000). At present, evidence has revealed that *NMS* and *NMU2R* mRNA are abundant in the central nervous system (hypothalamus), pituitary, and reproductive organs (testis) (Ma et al., 2017; Mori et al., 2005; Vigo et al., 2007; Yang et al., 2012). In addition, the *NMS* mRNA was highly expressed in the SCN, arcuate nucleus (ARC), and paraventricular nucleus (PVN) of the hypothalamus (Mori et al., 2005, 2012; van Esseveldt et al., 2000). In animals, the SCN, ARC, and PVN are key regions of the hypothalamus that regulate several physiological activities, such as circadian rhythm, hormone secretion, reproduction, etc. Moreover, the expression of NMS and NMU2R in the PVN indicates a putative role of NMS in regulation of the hypothalamic-pituitaryadrenal gland (HPA) axis (Kalra et al., 1999; Schwartz et al., 2000; Shousha et al., 2006). The currently available data on rats show that at least some neural systems that affect gonadotropin-releasing hormone (GnRH) release, and also control the *GnRH* gene expression in the hypothalamus (Ciechanowska et al., 2008). Intracerebroventricular (ICV) administration of NMS has evoked modest luteinizing hormone (LH) secretory responses in rats (Vigo et al., 2007). All these indicate that NMS may play roles in reproduction.

NMS and NMU2R were also identified in the pituitary gland, and exogenous NMS has been shown to inhibit LH secretion in ovariectomized (OVX) rats. Although it remains unclear how NMS neurons of the ARC regulate GnRH/LH secretion, the ARC has been involved in the induction of GnRH/LH surges, particularly in the regulation of leptin on LH (Anderson et al., 2002; Kaur et al., 2002). NMS is a confirmed participant in the central regulation of the gonadotropic axis by

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inhibiting LH responses to NMS after ICV administration in OVX rats (Peier et al., 2009; Quan et al., 2003). The expression of *NMS* and *NMU2R* mRNAs peaked in the proestrus phase of rat hypothalamus. However, decreasing levels of NMS and NMU2R by OVX were up-regulated by progesterone and not by 17-beta-E2; notably, an enhanced plasma LH concentration was inhibited by ICV NMS administration in OVX rats (Vigo et al., 2007). In addition, gonadotropin-inhibitory hormone (GnIH) and kisspeptins are master factors with pivotal roles as reproductive hormones. GnIH may regulate the reproductive behavior of animals though GnRH neurons directly (Bentley et al., 2003; Kriegsfeld et al., 2006). In contrast to GnIH, kisspeptins play an important role in regulation of gonadotropic axis, as positive regulator (Chianese et al., 2016; Meccariello et al., 2014). NMU2R (Ma et al., 2017) and GnIH (Wang et al., 2018) protein were mainly located in Leydig cells of pig testis. The androgen receptor (AR) plays a key role in the development of the testis by mediating actions of natural androgens, testosterone, and dihydrotestosterone (Gaba et al., 2018). There are several lines of evidence suggest that NMS may act on multiple components of the hypothalamic-pituitary-gonadal (HPG) axis. In addition, studies have shown that NMS can affect cell proliferation (e.g. porcine splenic lymphocytes) (Lin et al., 2016); therefore, NMS may stimulate the proliferation of porcine Leyding cells.

Except for rats and mice, pigs are also an important laboratory animal and important in animal husbandry. Previously, our lab had cloned the *NMS* and *NMU2R* genes of the pig and investigated their mRNA expression in female pig HPG axis during estrus cycle, and effect of NMS on female pig reproductive axis (Yang et al., 2009, 2010). Subsequently, we found that NMS and NMU2R were widely distributed in the hypothalamus, pituitary, and testis of male pigs, and undergo developmental changes in the HPT axis of the boar during the postnatal development stages (Ma et al., 2017); therefore, NMS might regulate the reproductive function in male pigs. To explore this hypothesis, we initially utilized immunocytochemistry to determine the distribution of NMU2Rs in hypothalamic cells, anterior pituitary cells and Leydig cells, where NMS may act to regulate the reproduction function. Subsequently, we investigated the effects of NMS on the secretion of LH, follicle-stimulating hormone (FSH), testosterone, and the expression of *NMU2R*, *GnRH*, *GnIH*, *LH*, *FSH* and steroidogenic mediators (*STAR*, *P450* and *3β-HSD*) after treatment with different doses of NMS (10^{-6}, 10^{-8}, 10^{-10}, and 10^{-12} M) (Lin et al., 2016; Yang et al., 2009) using radioimmunoassay and real-time PCR, respectively. Finally, we measured the effects of NMS on the expression of proliferating cell nuclear antigen (PCNA), Cyclin B1, as well as the colocalization of the NMU2R and AR or GnIH in Leydig cells using western blot and immunofluorescence staining, respectively. Taken together, these findings provide further evidence for the role of NMS in regulating reproduction in male pigs.

2. Materials and methods

2.1. Ethics statement

All the pigs were obtained from Jiangsu Academy of Agricultural Sciences and were fed according to the breeding standards of Chinese Local Pigs and the National Research Council (NRC). All experiments were performed according to the guidelines of the regional Animal Ethics Committee of Nanjing Agricultural University under project number 2009ZX08009-143B. Sample collection strictly complied with the Guidelines on the Ethical Treatment of Experimental Animals (2006) No. 398 set by the Ministry of Science and Technology, China. Throughout the experimental session, all the pigs were housed in a room at 22 ± 0.5 °C, 50 ± 5% humidity and a 12-h light/12-h dark cycle and were maintained with free access to a standard laboratory pellet diet and water. Each process was conducted in strict accordance with animal protection committee regulations to minimize the risks of injury.

2.2. Animals and drugs

Sixteen male Large White pigs (3 months old, weight 20 ± 2 kg at the start of the experiment) were used in this experiment. The pigs were anesthetized with intraperitoneal (i.p.) injection of 20% urethane (5 mL/kg body weight) and rapidly decapitated within 15 min after the anesthesia; the brains, anterior pituitaries, and testes were immediately removed and placed in ice-cold D-Hanks balanced salt solution that contained an antibiotic. All the pigs were free from overt signs of disease during the sample collection.

2.3. Tissue isolation and cell cultures

2.3.1. The isolation and culture of pig hypothalamic cells

The hypothalamus was separated according to previously described methods (Boyadjieva and Sarkar, 2010; Sarkar et al., 2008). The viability and concentration of hypothalamic cells were determined by Trypan blue exclusion in hemacytometer, and cell suspensions with 95% or greater viability were planted in 6-well cell culture plates (1.5×10^6 cells/well) in Dulbecco's modified Eagle's medium/Ham F12 50/50 mix (DMEM/F12, Wisent INC, Canada) that contained 15% fetal bovine serum (FBS) and 1% penicillin/streptomycin. After 48 h, the culture medium was replaced with DMEM/F12 that contained 10% FBS and 3 μg/mL of cytosine arabinoside (Sigma) to prevent the overgrowth of the glial cells. The cells were maintained for 24 h in this medium. On day 4, the medium was replaced with medium that contained different doses of NMS (10^{-6}, 10^{-8}, 10^{-10}, and 10^{-12} M) and a vehicle control. Prior to adding NMS, We cultured 6 wells from these cells were fixed for immunocytochemical staining of NMU2R. Incubated NMS for 24 h later, the medium was collected and stored at −20 °C until the cells were harvested for real-time PCR.

2.3.2. The isolation and culture of pig anterior pituitary cells

The anterior pituitary cells were dispersed aseptically according to previously described methods (Bogacka et al., 2002; Szafranska and Tilton, 2000). The viability and concentration of the cells were determined by Trypan blue exclusion in a hemacytometer, and the cell suspensions with 95% or greater viability were planted in 6-well plates (1×10^6 cells/mL) in DMEM that contained 15% FBS and 1% penicillin/streptomycin and were incubated for 3 days at 37 °C in a water-saturated atmosphere that contained 5% CO_2. The medium was replaced with DMEM that contained NMS at a concentration of 10^{-6}, 10^{-8}, 10^{-10}, and 10^{-12} M and a vehicle control. Prior to adding NMS, We cultured 6 wells from these cells were fixed for immunocytochemical staining of NMU2R. After incubated NMS for 24 h, the medium was collected and stored at −20 °C until LH and FSH were measured using a radioimmunoassay. The cells were harvested for real-time PCR.

2.3.3. The isolation and culture of pig Leydig cells

The testes were separated according to the methods described by Raeside et al. (2006) (Lervik et al., 2011; Raeside et al., 2006). Leydig cells were purified by centrifugation through a discontinuous Percoll gradient. Percoll (GE Healthcare Life Sciences, USA) was made *iso*-osmotic by the addition of 1 volume of 1.5 M NaCl to 9 volumes of Percoll. This 90% Percoll was further diluted with 0.15 M NaCl to generate 60%, 34%, 26%, and 21% Percoll solutions. The four types of Percoll solutions were layered to form the gradient and centrifuged at 250 × g for 30 min at 4 °C. The enriched Leydig cell fraction was harvested from the 34% layer and 60% layer, washed, filtered, and counted in hemacytometer. The purity of the Leydig cells after the gradient centrifugation, as determined by immunocytochemistry for 3β-HSD (Ma et al., 2018), was greater than 90%. The viability and concentration of the cells were determined by Trypan blue exclusion in a hemacytometer, and cell suspensions with 95% or greater viability were planted in 6-well cell culture plates (1.5×10^6 cells/well) in DMEM/F12

Table 1
Primers and annealing temperature for real-time PCR.

Genes	Primer sequence (5-3′)	Annealing (°C)	efficiencies values(%)	Accession Nos.
NMUR2	(+)CTCCACGGCATCAAGTTCCA (−)CCACATGGGCTTGATGACTGTAC	58	102.358	XM_003134131
GnRH	(+)GAGCCAATTCCGAAACTTCTAGC (−)CTTCTGCCCAGTTTCCTCTTCA	54	106.289	NM_214274.1
GnIH	(+)GTTCCCAATCTGCCCCAAAG (−)CGTGGATTGTTGGAGCAAAGC	58	99.022	HQ000001.2
LHβ	(+)TGTTGTGGCTGCTGCTGAG (−)GATGCTGGTGGTAAAGGTGATG	60	100.353	NM_214080.1
FSHβ	(+)TCAGTCCTATCACCACTCAACC (−)CTTAGCCCATTTCCTTCCTC	60	96.191	NM_213875.1
3β-HSD	(+)GGAGGAAGCCAAGCAGAAAA (−)TTTTCAGCGCCTCCTTGTG	58	99.305	NM_001004049
STAR	(+)AGAGCTTGTGGAGCGCATG (−)CATGGGTGATGACTGTGTCTTTTC	60	99.301	NM_213755
P450	(+)AGCCAAGACGAACGCAGAA (−)CCCCAAAGATGTCCGCAAC	60	98.736	NM_214427
β-actin	(+) CTCCATCATGAAGTGCGACGT (−)GTGATCTCCTTCTGCATCCTGTC	60	98.899	XM_003124280.2

(Wisent INC, Canada) that contained 5% FBS and 1% penicillin/streptomycin. After 48 h, the culture medium was replaced with DMEM/F12 that contained 1% penicillin/streptomycin. The cells were maintained for 24 h in this medium. On day 4, the medium was replaced with a medium that contained different doses of NMS (10^{-6}, 10^{-8}, 10^{-10} and 10^{-12} M) and a vehicle control. Prior to adding NMS, We cultured 6 wells from these cells were fixed for immunocytochemical staining of NMU2R. After incubated NMS for 24 h, and the medium was collected and stored at −20 °C until the testosterone levels were measured by radioimmunoassay. The cells were harvested for real-time PCR and western blot.

2.4. Immunocytochemistry (ICC)

The cells were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (0.1 M PBS, pH 7.2) for 30 min and were subsequently washed three times with PBS. The procedure for immunocytochemistry has previously been described (Yao et al., 2009). The cells were immersed in 0.3% H_2O_2 in methanol for 30 min at room temperature. The cells were then incubated with 5% BSA for 30 min. The cells were subsequently incubated with a NMU2R antibody (rabbit anti-NMU2R polyclonal antibody, GTX71489, GeneTex diluted 1:200 in 0.1 M PBS) for 24 h at 4 °C. Following rinsing with 0.1 M PBS for 5 min three times, the slides were subsequently incubated with the secondary antibody (goat-anti-rabbit IgG, Wuhan Boster Biological Technology Co. Ltd., China) for 1 h at 37 °C. After rinsing with PBS, the sections were placed in a streptavidin-biotin complex (Wuhan Boster Biological Technology Co. Ltd., China) for 30 min and were washed again in 0.1 M PBS. The signals were developed using a freshly prepared 0.02% DAB (Wuhan Boster Biological Technology Co. Ltd., China) in distilled water for approximately 20 min. The sections were counterstained with hematoxylin. All the sections were subsequently dehydrated, mounted and covered with glass coverslips. In the negative control, the primary antibody was replaced with normal goat serum. The images were acquired using bright-field microscope (Olympus BH-2, Olympus China Ltd. Corp., Beijing, China).

2.5. Radioimmunoassay (RIA)

The concentrations of the hormones LH, FSH and testosterone in the culture media were measured by RIA. The hormone levels in the media were measured in a volume of 50 μL using a double-antibody method and commercial RIA kits purchased from Biomedicals (BNIOBT, China) according to the manufacturer's instructions. All the hormones were labeled with iodine-125 (^{125}I) using the chloramine-T method, and the concentrations were determined using the reference preparation hormones as the standards. The intra- and inter-assay coefficients of variation were 4.8% and 9.0% for LH, 4.5% and 10.2% for FSH, and 8.5% and 10% for testosterone, respectively. The assay sensitivity was 0.2 mIU/mL for LH, 0.2 mIU/mL for FSH, and 0.02 ng/mL for testosterone.

2.6. Relative real-time PCR

For all the samples, the total RNA extraction, reverse transcription reaction and real-time PCR reactions were performed, as previously described (Li et al., 2013). Briefly, total RNA was extracted using the TRIzol extraction method (TRIzol reagent, TaKaRa, Japan). Then the RNA concentration and quality were detected using a photometer (Eppendorf Biophotometer, Germany), and the D260/D280 ratios were showed at 1.8–2.0. The RNA integrity was verified by agarose gel electrophoresis andethidium bromide staining, and the 18S and 28S rRNA bands were separated without a leading smear. For each sample, equal amounts of all RNA samples were simultaneously reverse transcribed using an oligo (deoxythymidine) 15 primer and M-MLV reverse transcriptase (TaKaRa, Japan), according to the manufacturer's instructions. All reverse transcriptase reactions were performed at 42 °C including a negative control, which contained nuclease-free water instead of RNA.

All real-time PCR were conducted using an Applied Biosystems 7500 Sequence Detector (Applied Biosystems, CA) with the SYBR® Green Master Mix Kit (Vazyme Biotech Co., Ltd, China). The primer sequences for the genes are shown in Table 1. The pig *β-actin* gene was used as an internal control. The PCR reactions were conducted using the following program: 94 °C for 5 min, followed by 40 cycles of amplification with denaturation at 95 °C for 30 s and annealing temperature (shown in Table 1) for 30 s. Melting-curve analysis was recorded to verify a single-product generation at the end of the reaction. The mRNA quantities of the different genes were expressed as a proportion of the *β-actin* mRNA quantity using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.7. Western blot

The Leydig cells were washed with cold PBS and lysis buffer containing 50 mM of Tris-HCl, pH 7.4, 2% SDS, 10 mM of dithiothreitol (DTT), and 5 mM of EDTA with supplements of proteinase inhibitors. Equal amounts of the proteins (40 μg) were fractionated on a 12% SDS-PAGE, and the separated proteins were transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 5% bovine serum albumin (BSA) and were subsequently incubated with

primary antibody at the appropriate dilution (PCNA (sc-7907, dilution 1:200, Santa Cruz); Cyclin B1 (sc-595, dilution 1:200, Santa Cruz) and GAPDH (dilution 1:1000, Santa Cruz)) (Li et al., 2013; Ma et al., 2018). Following incubation with horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody (dilution 1:1000, Boster), the immunoreactive proteins were detected using an ECL chemiluminescent chromogenic kit (Vazyme). The membranes were subsequently exposed to X-ray film (Fuji Photo Film Co., Ltd., Tokyo, Japan). Densitometric quantification was performed using Image J (National Institutes of Health, Bethesda, MD) with GAPDH as the internal control for normalization.

2.8. Dual-labeling immunofluorescence staining

2.8.1. NMU2R and AR

The Leydig cells were fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.2) for 30 min, washed three times with PBS and incubated with 0.4% Triton X-100 in 0.1 M PBS for 20 min at room temperature, immersed in 0.3% H_2O_2 in methanol for 30 min, and then incubated with 5% BSA for 30 min. The cells were subsequently incubated with the NMU2R antibody (rabbit anti-NMU2R polyclonal antibody, GTX71489, GeneTex diluted 1:200 in 0.1 M PBS) at 4 °C overnight, then washed three times in 0.1 M PBS and incubated for 8 h at 4 °C with rhodamine (TRITC)-conjugated Affini Pure Donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). The excitation wavelength for the TRITC-induced fluorescence was 568 nm. The cells were subsequently washed five times and incubated with the anti-AR antibody (goat polyclonal; ab19066, Abcam, USA) 1:200 in 0.1 M PBS at 4 °C overnight, then washed 3 times in 0.1 M PBS and incubated for 8 h at 4 °C with fluorescein (FITC)-conjugated Affini Pure Donkey anti-goat IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). The excitation wavelength for the FITC-induced fluorescence was 488 nm. The cells were washed five times and were incubated for 5 min with DAPI (in 4, 6-diamidino-2-phenylindole), and then washed 3 times, coverslipped with glycerol, sealed and analyzed under a confocal microscope.

2.8.2. NMU2R and GnIH

The procedure for NMU2R and GnIH was similar to that of NMU2R and AR procedure (Section 2.8.1.). The cells were subsequently washed five times and were incubated with the anti-GnIH antibody (guinea pig anti-human neuropeptide VF, catalog No. N217647, Biological, USA; diluted 1:400 in 0.1 M PBS) at 4 °C overnight.

2.9. Statistical analysis

To identify significant differences, the data were analyzed using GraphPad Prism 5. All data were presented using the means ± standard error of the mean (SEM). The statistical analysis was performed using one-way ANOVA and the multiple comparisons test (Tukey's) with SPSS Statistics 19.0 (SPSS, IBM®, NY, USA).

3. Results

3.1. Effects of NMS on NMU2R, GnRH and GnIH mRNA expression

NMS may regulate reproduction in the HPG axis by activating NMU2R; thus, we initially investigated the localization of the NMU2R in pig hypothalamic cells to determine whether NMS was able to activate its receptors in pig hypothalamic cells. ICC results indicated that the NMU2R was predominately localized in the cell body and dendrites of hypothalamic cells according to the brown staining of the NMU2R and the blue staining of the nuclei (the nuclei were counterstained with hematoxylin) (Fig. 1A). No immunoreactivity was identified in the control sections from which the primary antibody was omitted for the incubation (Fig. 1B).

To investigate whether exogenous NMS affected the release of GnRH and its underlying mechanism, we measured the expression of *NMU2R*, *GnRH*, and *GnIH* mRNA in hypothalamic cells treated with different doses of NMS. Compared with the control group, different doses of NMS increased the expression of *NMU2R* mRNA; the *NMU2R* expression significantly increased when the cells were treated with 10^{-8} and 10^{-10} M NMS ($P < 0.01$), and at 10^{-8} M, the expression level of *NMU2R* mRNA was the peak (Fig. 1C). Similar to the result of *NMU2R*, different doses of NMS also increased the expression of *GnRH* mRNA compared with that of the control group; Except 10^{-12} M, 10^{-6} M NMS increased the expression of *GnRH* ($P < 0.05$), and at 10^{-8} and 10^{-10} M doses similarly affected *GnRH* expression ($P < 0.01$) (Fig. 1D). As shown in Fig. 1E, the mRNA levels of *GnIH* were reduced after treatment with different doses of NMS; and the significant decrease in *GnIH* occurred when the cells were treated with 10^{-6} and 10^{-8} M NMS ($P < 0.01$).

3.2. Effects of NMS on gonadotropin secretion and the expression of NMU2R, LH and FSH mRNA

As shown in Fig. 2A, the NMU2R was detected in anterior pituitary cells, and the density of NMU2R-immunoreactivity was observed. There was no immunoreactive staining in the negative control (Fig. 2B). These results suggested that NMS might affect the secretion of gonadotropin in anterior pituitary cells through the NMU2R.

To study whether exogenous NMS affects the secretion of gonadotropin, we investigated LH and FSH concentrations in the incubation medium of primary anterior pituitary cells after treatment with different doses of NMS. Compared with the control group, the concentration of LH significantly increased when the cells were treated with 10^{-8} M and 10^{-10} M NMS ($P < 0.05$), and at 10^{-8} and 10^{-10} M doses had the similar effects; however, there was no significant effect on LH secretion when the cells were treated with doses of NMS (10^{-6} and 10^{-12} M) (Fig. 2C). In addition, compared with the control group, the concentration of FSH increased significantly when the cells were treated with 10^{-8} M and 10^{-12} M NMS ($P < 0.01$). No significant increase occurred at doses of 10^{-6} M and 10^{-10} M doses (Fig. 2D). These results showed that certain doses of NMS might promote the secretion of LH and FSH in primary anterior pituitary.

NMS exerts its effect by binding to NMU2R; therefore, we further investigated the expression of *NMU2R* mRNA in primary anterior pituitary cells after treating with different doses of NMS. As shown in Fig. 2E, compared with the control group, real-time PCR results showed that 10^{-8} M NMS could significantly increase the expression of *NMU2R* ($P < 0.05$); in this experiment. 10^{-6} M NMS ($P < 0.01$), 10^{-8} M NMS ($P < 0.01$), 10^{-10} M NMS ($P < 0.01$) and 10^{-12} M NMS ($P < 0.05$) significantly increased the expression of *LH* mRNA compared with the control group (Fig. 2F). Except 10^{-6} M, 10^{-8} M NMS ($P < 0.01$), 10^{-10} M NMS ($P < 0.05$), 10^{-12} M NMS ($P < 0.01$) significantly increased the expression of FSH compared with that of the control group and the highest expression of *FSH* was identified in the cells treated with the dose of 10^{-8} M NMS (Fig. 2G). Taken together, these results indicated that NMS/NMU2R system might modulate gonadotropin synthesis by activating LH and FSH in primary anterior pituitary.

3.3. Effects of NMS on testosterone secretion, and the expression of NMU2R, STAR, P450 and 3β-HSD mRNA

We investigated the localization of the NMU2R in Leydig cells from pig testes. Leydig cells showed NMU2R positive immunoreactivity (Fig. 3A), and no immunoreactivity was identified in the control sections (Fig. 3B). This result indicated that NMS might affect testicular function through the Leydig cells.

To study whether exogenous NMS affects the secretion of testosterone, we investigated testosterone concentration in the Leydig cells treated with different doses of NMS. Compared with the control group,

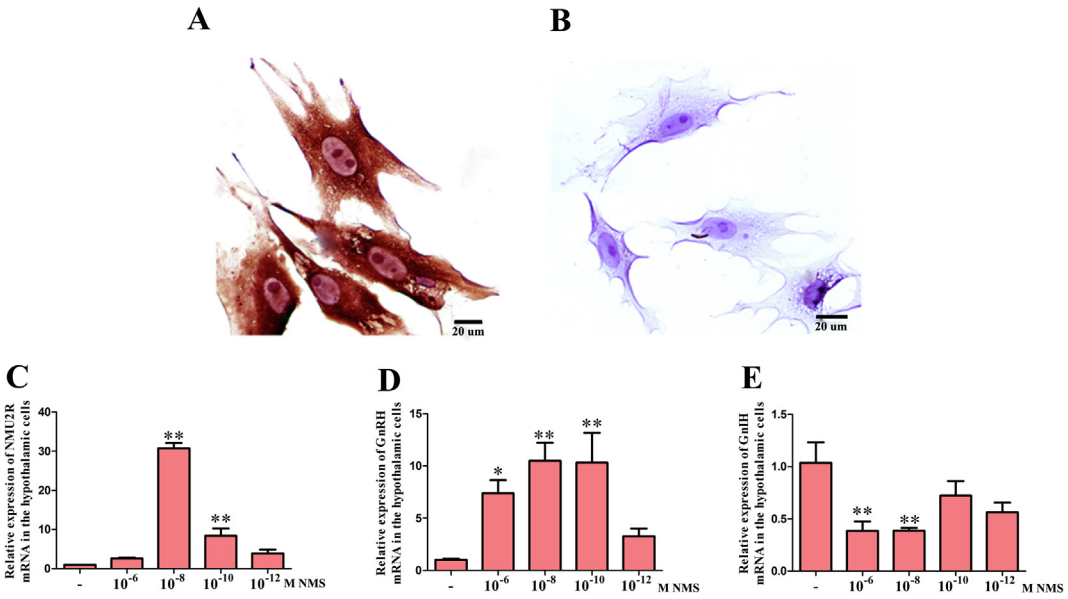

Fig. 1. Immunohistochemical localization of NMU2R protein, and the effects of NMS on NMU2R, GnRH and GnIH mRNA expression in hypothalamic cells. (A) Strong immunoreactivity of the NMU2R protein was localized in the hypothalamic cells. (B) Negative control shows no immunostaining. (C, D and E) The expression patterns of NMU2R, GnRH and GnIH mRNA in the cultured hypothalamic cells with various doses of NMS treatments (n = 4). "−" as the control group. Values are means (± SEM). Compared with control group, asterisk indicates significant difference. $^{*}P < 0.05$, $^{**}P < 0.01$. Scale bars in (A and B) = 20 μm.

10^{-8} M NMS significantly increased the testosterone concentration ($P < 0.01$); and 10^{-6} M, 10^{-10} M and 10^{-12} M NMS showed no significant increase (Fig. 3C).

Subsequently, we further investigated the expression of *NMU2R* and steroidogenic mediators (*STAR*, *P450* and *3β-HSD*) mRNA in primary Leydig cells after treating with different doses of NMS. Real-time PCR analysis indicated that the doses of 10^{-6} M and 10^{-8} M NMS induced a significantly ($P < 0.01$) increased expression of *NMU2R* compared with that of the control (Fig. 3D). The expressions of *STAR* were increased at doses of 10^{-6} M and 10^{-10} M NMS ($P < 0.05$, Fig. 3E), the *P450* were significantly increased at doses of 10^{-6} M and 10^{-10} M NMS ($P < 0.01$, Fig. 3F). Moreover, all the doses of NMS used in this experiment could significantly stimulate the expression of *3β-HSD* ($P < 0.01$, Fig. 3G). Taken together, these results indicate that the NMS/NMU2R system might promote testosterone production by regulating the expression of STAR, P450 and 3β-HSD in primary Leydig cells.

3.4. Effects of NMS on PCNA and Cyclin B1 protein

As shown in Fig. 4A, PCNA and Cyclin B1 immunoblots showed a single band at ~36 kDa and ~55 kDa respectively. Densitometric analysis of the western blots indicated a marked variation in the expression of all the proteins when the Leydig cells were treated with different doses of NMS. All the doses of NMS induced an increase in the expression of PCNA compared with that of the control; the doses of 10^{-8} M and 10^{-10} M NMS induced a significantly increased expression of PCNA (Fig. 4B, $P < 0.05$). However, only 10^{-6} M NMS showed a sharp increase in the Cyclin B1 protein expression ($P < 0.05$); the other doses of NMS showed no effect on the Cyclin B1 protein expression (Fig. 4C).

3.5. NMU2R colocalizes with AR or GnIH

As shown in Fig. 5, the immunofluorescence study results demonstrated that NMU2R, GnIH and AR all localized in Leydig cells. GnIH protein expression was identified in the cytoplasm of Leydig cells, which colocalized with the NMU2R (Fig. 5A, B, C and D). In addition, AR protein expression was identified in the nucleus and less in the cytoplasm of Leydig cells (Fig. 5E, F, G and H). All these results suggested that NMU2R, GnIH and AR colocalized in Leydig cells.

4. Discussion

The aim of this study was to localize the NMU2R in hypothalamic cells, anterior pituitary cells and Leydig cells; to determine whether NMS was able to alter LH, FSH, and testosterone secretion; and further to examine the expression of relative genes. Moreover, this work aimed to further support the role of this peptide in male pigs by detecting NMS to control the proliferation in Leydig cells *in vitro* and the colocalization between NMU2R and AR or GnIH. The results suggested that NMS played a role in reproduction in male pigs. The conclusion is further supported by the finding of altered LH levels by the central administration of NMS (Vigo et al., 2007; Yang et al., 2010). As indicated in introduction, the SCN, ARC, and PVN are key regions of the hypothalamus that regulate several physiological activities in animals. In the rat hypothalamus, *NMS* mRNA is predominately expressed in the SCN, with very slight expression in other brain regions (Mori et al., 2005). However, the distribution and location of NMS-positive cells have mainly been identified in pig hypothalamic region, including the periventricular nucleus (PEN), PVN, SCN, supraoptic nucleus (SON), ventromedial nucleus (VMH) and ARC (Yang et al., 2012). Furthermore, NMS and NMU2R were widely distributed in the central nervous system, pituitary, and testis, which suggested that NMS might play a reproductive role in the Xiaomeishan boar (Ma et al., 2017). The evidence of GnRH neurons widely contained in the hypothalamic PVN and ARC (Campbell et al., 2003; Ciechanowska et al., 2008; Kumar et al., 1991) corresponded to a decreased LH level in mammals after treatment with NMS (Vigo et al., 2007; Yang et al., 2010), which indicated that NMS may play an important role in the regulation of reproductive function via the NMU2R or GnRH.

To validate this hypothesis, we systemically characterized the direct effects of NMS on the male pig hypothalamic cells, anterior pituitary cells and Leydig cells *in vitro*. ICC results showed that NMU2R widely distributed in male pig hypothalamic cells, anterior pituitary cells and Leydig cells suggesting that the NMS/NMU2R system existed in male pig reproductive axis and may play a significant role in the regulation of gonadotropin secretion in the brain and testis, which was similar to its

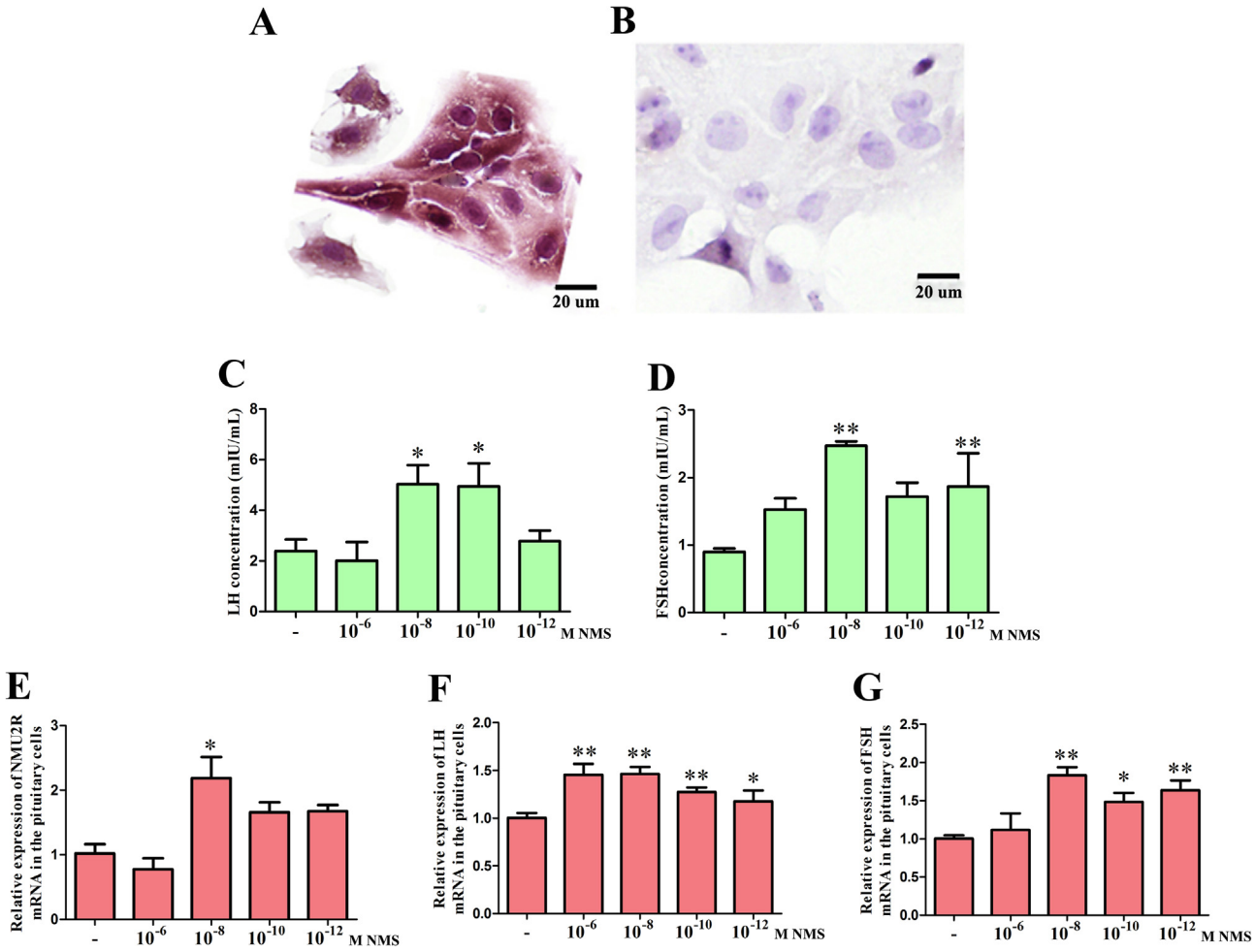

Fig. 2. Immunohistochemical localization of NMU2R protein, as well as the effects of NMS on gonadotrophin secretion and NMU2R, LH and FSH mRNA expression in pituitary cells. (A) Strong immunoreactivity for NMU2R was observed in pituitary cells. (B) Negative control showed no immunoreactivity. (C and D) The stimulatory effects of NMS on LH and FSH secretion in the cultured pituitary cells (n = 4). (E, F and G) The effects of NMS on NMU2R, LH and FSH mRNA expression in the cultured pituitary cells (n = 4). "−" as the control group. Values are means (± SEM). Compared with control group, asterisk indicates significant difference. $^{*}P < 0.05$, $^{**}P < 0.01$. Scale bars in (A and B) = 20 μm.

function in other mammals. In male pig hypothalamic cells, we found that NMS could promote the expression of *GnRH* mRNA and restrain the expression of *GnIH* mRNA using real-time PCR. Previously, Guihong Yang et al. had clarified that NMS had a stimulatory effect on GnRH release *in vitro*, which suggested that NMS could regulate GnRH activities in female pig hypothalamus (Yang et al., 2009). In addition, we also found that NMS could increase the expression of *NMU2R* mRNA in male pig hypothalamic cells by real-time PCR. It's well known that NMS play roles by binding to NMU2R; therefore, we speculate that NMS/NMU2R system might also regulate GnRH activities in male pig hypothalamus, but the specific regulatory mechanism remains to be elucidated.

Previous researches and the current findings demonstrated that NMS could stimulate LH release, which indicated that NMS directly regulate reproduction in animals (Vigo et al., 2007; Yang et al., 2009). In male pig anterior pituitary cells, our findings showed that the level of LH significantly increased in pituitary cells treated with 10^{-8} M NMS, consistent with previous studies. In addition, the change of FSH level was similar with that of LH in pituitary cells. Subsequently, we found that NMS could promote the expression of *LH* and *FSH* mRNA in primary male pig anterior pituitary cells. These results showed that NMS may have a regulatory effect on the secretion of gonadotropin (LH and FSH) in male pig pituitary. Although the expression profiles of *LH* and *FSH* mRNA do not fully parallel the release of LH and FSH. There are some possible reasons causing the discrepancies. First, both LH and FSH are glycoprotein hormones produced by basophils in the anterior pituitary gland, post transcriptional modification may induce inconsistency between LH/FSH mRNA and the LH/FSH level in the culture media. Second, different doses treatment of NMS may change the stability of proteins. Last, different doses treatment of NMS caused some modification which affect the recognition in radioimmunoassay. However, further study is needed to illuminate the discrepancies between mRNA expression and LH and FSH synthesis under NMS treatment.

Our previous and present studies confirm that NMU2R distributed in Leydig cells and different doses of NMS could regulate testosterone synthesis and release from Leydig cells. In addition, the colocalization between the NMU2R and AR or GnIH indicated that NMS may play a role via NMU2R to mediated AR or GnIH. Previous researches showed that AR plays important regulatory roles in the male reproductive system, and the function of AR has primarily been attributed to transcriptional stimulation of target genes via androgen response elements located in their promoters and enhancer (Baniwal et al., 2009). Recently, both GnIH and GnIH-R were expressed in germ cells (spermatocytes and spermatids) in the testis, which has suggested the direct action of GnIH on Leydig cells via GnIH-R in avian gonads (Bentley et al., 2008). Basal testosterone levels in primary cultures of porcine Leydig cells have previously been reported (Lervik et al., 2011). Lervik et al. determined that Leydig cells treated with LH could induce

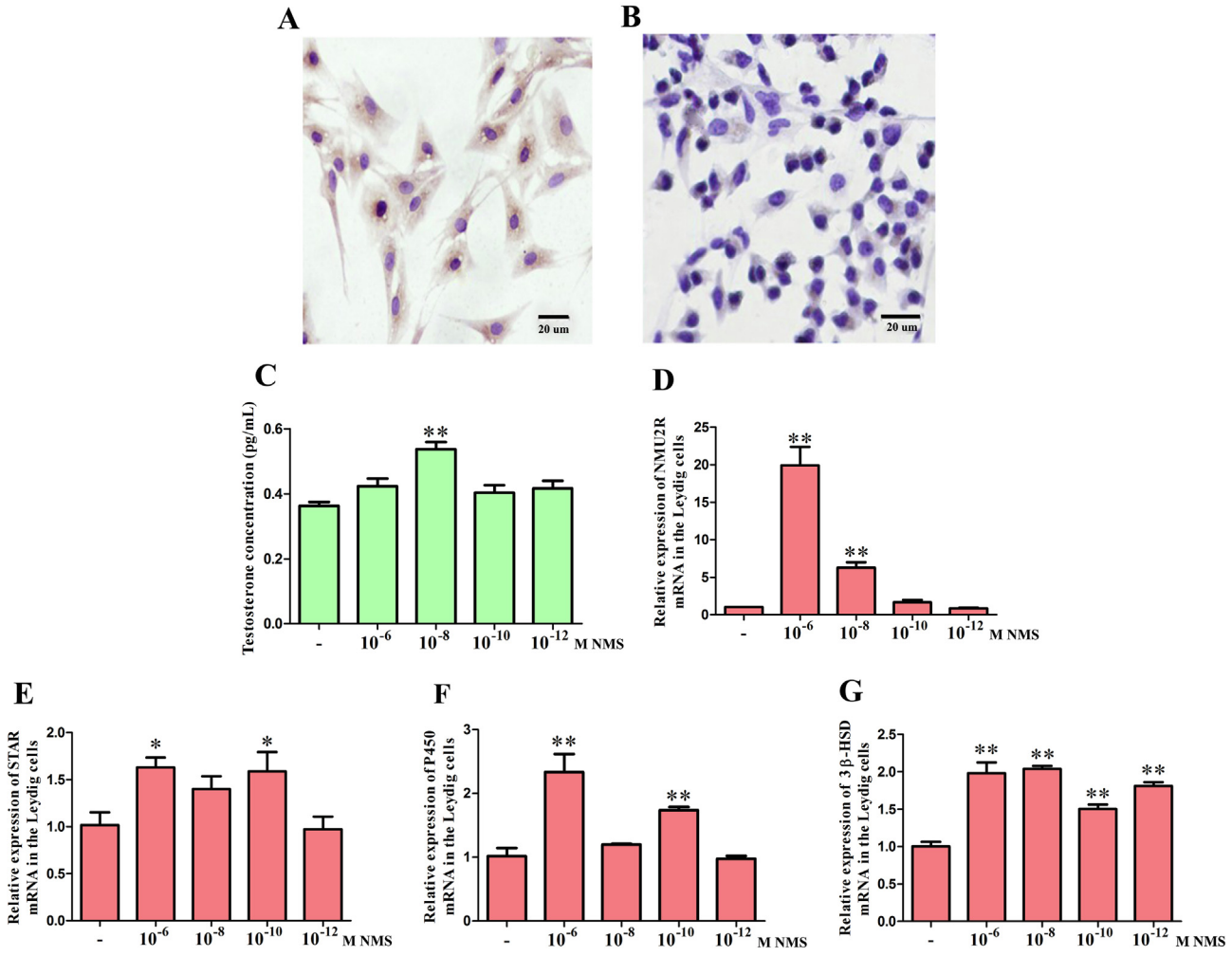

Fig. 3. Immunohistochemical localization of NMU2R protein, and the effect of NMS on testosterone secretion and the mRNA expression of NMU2R, STAR, P450 and 3β-HSD in Leydig cells. (A) Strong immunoreactivity for NMU2R was observed in the cytoplasm of Leydig cells. (B) Negative control showed no immunoreactivity. (C) The stimulatory effect of NMS on testosterone secretion (n = 4). (D, E, F and G) The stimulatory effects of NMS on NMU2R, STAR, P450 and 3β-HSD mRNA expression in the cultured Leydig cells (n = 4). "−" as the control group. Values are means (± SEM). Compared with control group, asterisk indicates significant difference. $^{*}P < 0.05$, $^{**}P < 0.01$. Scale bars (A and B) = 20 μm.

testosterone synthesis and release (Lervik et al., 2011). Despite accumulating data suggested that NMS affects gonadal steroid secretion, there has been limited information regarding the mechanism of NMS effect testis function to date. Moreover, we found that certain dose of NMS promote the expression of *STAR*, *P450* and *3β-HSD* mRNA. These results provide a theoretical basis for further research on the reproductive function of NMS in male pigs.

Additionally, the Leydig cells treated with NMS indicated that NMS up-regulated the expression of the cell cycle proteins PCNA and Cyclin B1. The expression of PCNA and Cyclin B1 is associated with the proliferation and transition of testis cells through the late G1 and S phases of mitosis. Therefore, our data suggested that NMS increased cell proliferation in Leydig cells by impacting these phases of the cell cycle (Altay et al., 2003; Chang et al., 2003; Nurse, 2000). This effect may induce testis growth and development. In the testis, PCNA was expressed in spermatogonia and early-phase primary spermatocytes of the seminiferous tubules (Shi et al., 2015), which are also associated with changes in cellular proliferation and cell cycle protein expression. Collectively, these findings indicate that NMS might promote the proliferation in pig Leydig cells, while the specific mechanism should be further studied.

5. Conclusion

The results provide evidence that NMS has a direct effect on the HPT axis in male pigs *in vitro*. NMS is a multifunctional factor, which regulates the synthesis and release of gonadotrophin releasing hormone, gonadotrophin and steroid hormone and is involved in Leydig cell proliferation. To our knowledge, this investigation is the systemic study to show that NMS has a direct effect on the reproductive axis *in vitro* in male pigs. A better understanding of the independent actions of NMS at different levels of the reproductive axis enables us to understand and manipulate vertebrate reproduction from an entirely new perspective.

Conflict of interest

The authors have nothing to disclose regarding potential conflicts of interest, financial or otherwise.

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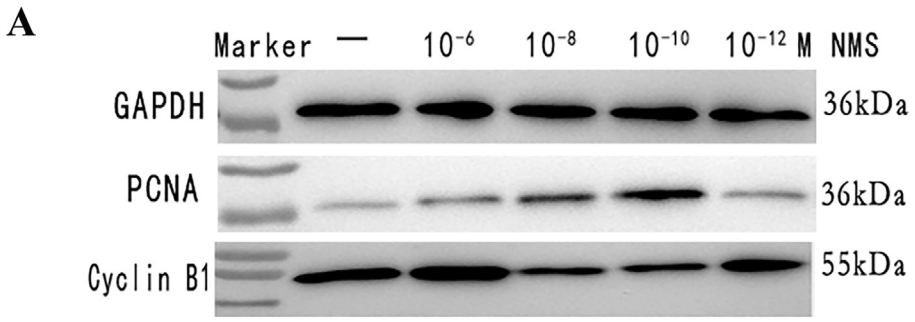

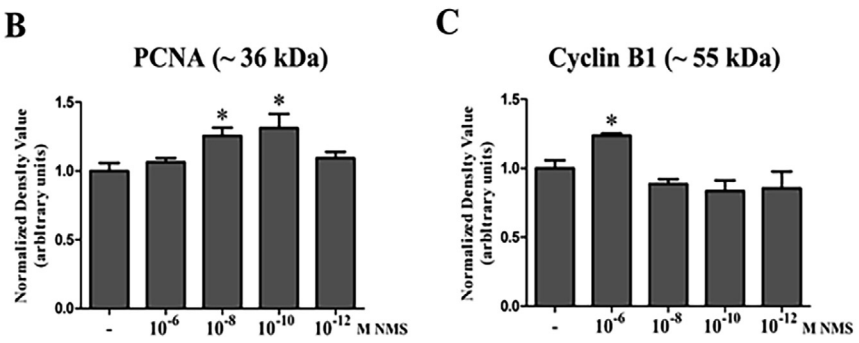

Fig. 4. Effects of NMS on PCNA and Cyclin B1 protein expression in Leydig cells (n = 4). (A) The stimulatory effects of NMS on PCNA and Cyclin B1 protein expression in the cultured Leydig cells was detected by western blot analysis. (B and C) Normalized density value of the PCNA and Cyclin B1 expression by gray analysis. "Marker" as protein ladder (26616, thermo scientific); "−" as the control group. Values are means (± SEM). Compared with control group, asterisk indicates significant difference. $^{*}P < 0.05$.

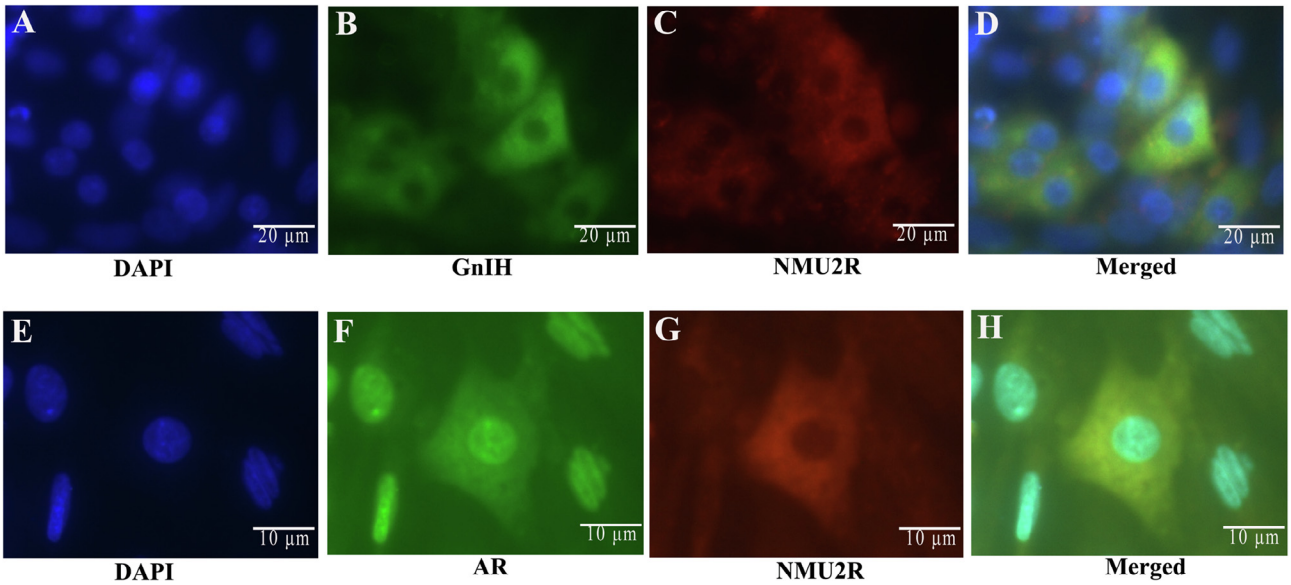

Fig. 5. Immunofluorescence of GnIH/AR and NMU2R protein in Leydig cells. (A and E) Nuclei (blue) staining of DAPI in the cultured Leydig cells. (B and F) Staining of GnIH and AR protein in the cultured Leydig cells (green). (C and G) Staining of NMU2R protein in the cultured Leydig cells (red). (D) A merged image of A, B and C; (H) A merged image of E, F and G. Scale bars (A, B, C and D) = 20 μm, (E, F, G and H) = 10 μm.

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