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NEUROGENIN3 REGULATES *SOHLH1* DURING SPERMATOGNESIS IN MICE

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ABSTRACT

Spermatogonial stem cells (SSCs) are tissue-specific stem cells in testis, and differentiation of SSCs contributes to spermatogenesis and male fertility. However, little is known about factors regulating spermatogonial differentiation. *Neurogenin3* (*Ngn3*), a class B basic helix-loop-helix (bHLH) transcription factor, recognized for its role in promoting the development of neurons in the hypothalamus, specifically expresses in undifferentiated spermatogonia and is a critical differentiation factor of mammalian stem and progenitor spermatogonia. *Sohlh1* (spermatogenesis and oogenesis specific basic helix-loop-helix 1) is regarded as a key factor in spermatogenesis, which promotes the expression of *Kit*, the marker of differentiated spermatogonia. In this study, we firstly confirmed that the expressionlevelof*Ngn3* is decreased in *Sohlh1*$^{-/-}$ mice by real-time PCR and western blot, and revealed the associationbetween*Ngn3*and *Sohlh1*. We then showed that *Ngn3* could bind to the promoter of *Sohlh1*andincrease the transcription of *Sohlh1* by ChIP and luciferase reporter gene assay. Lastly, we demonstrated that the protein of *Ngn3* and *Sohlh1* could bind together in wild type testis by immune precipitation. In conclusion, our study showed the association between *Ngn3* and *Sohlh1* for the first time. *Ngn3* may regulate spermatogenesis by binding with *Sohlh1*, which may be the direct target of *Ngn3* in differentiation of SSC.

Key-words:*Ngn3; Sohlh1*; spermatogenesis; transcription factor.

INTRODUCTION

The basic helix–loop–helix (bHLH) transcription factors of the Neurogenin (Ngn) family are positive regulators of neurogenesis (Kageyama and Nakanishi, 1997) and inhibitors of gliogenesis(Nieto *et al*., 2001; Sun *et al*., 2001). Basic-helix-loop-helix (bHLH) transcriptional regulators control both the determination and the differentiation of specific cell types in a variety of tissues, including muscle and nerve(Garrell and Campuzano, 1991). The three neurogenins (Ngn1, 2, and 3) form a subfamily of atonal-like sequences, which control early determination steps and then activate neuro D in each of the regions where they are expressed (Ma *et al*., 1996).

Ngn3 is also expressed in testis, specifically in As, Apr, and Aalspermatogonia (Yoshida *et al*., 2004). Spermatogonia are adult germline stem cells that can both self-renew and differentiate into spermatocytes. Differentiation of spermatogonia contributes to spermatogenesis and male fertility. Usually A1 to A4, Intermediate, and Type B spermatogoniaarenamed differentiated spermatogonia, whereas As, Apr, and Aalspermatogonia are undifferentiated spermatogonia. As spermatogoniais thought as spermatogonial stem cells (SSC) while Apr and Aal cells show virtually veryfewdifferentiation characteristics. Apr and Aalspermatogonia in seminiferous epithelium are actually differentiating cells in the sense that they are irreversibly committed to take further developmental steps in the direction of spermatocytes, which are also called spermatogonial progenitors(Phillips *et al*., 2010). Little is known about factors regulating spermatogonial differentiation, especially the differentiation of undifferentiated spermatogonia. *Ngn3* has been well-known as a marker of undifferentiated spermatogonia, which delineates the earliest stages of spermatogenesis in the mouse testis(Kaucher *et al*., 2012; Yoshida *et al*., 2004) has demonstrated that *Ngn3* is a critical downstream effector for *STAT3*-regulated differentiation of mammalian stem and progenitor spermatogonia. However, the downstream effector of *Ngn3* is still unclear.

Sohlh1(spermatogenesis and oogenesis specific basic helix-loop-helix 1) is regarded as a key factor in spermatogenesis. Loss of *Sohlh1* causes male infertility by disrupting spermatogonial differentiation into spermatocytes. Ballow *et al*. (2006) reported that *Ngn3* expression decreased in *Sohlh1*knockout mice, indicating that there may be association between *Ngn3* and *Sohlh1*. In this study, we analyzed the association between*Ngn3* and *Sohlh1* in testis by ChIP and other experiments.

MATERIALS AND METHODS

Materials: Human embryonic kidney cells (HEK293T) and C57BL/6J mice were supplied by the Laboratory Animal Centre of China Medical University, China. *Sohlh1* knockout mouse was a gift of Professor Wei Yan (Department of Cell Biology and Physics, University of Nevada, Reno, USA). All animals were housed in a barrier facility under 12 hour light and 12 hour dark conditions with free access to food and water. All procedures were approved by the IACUC of China Medical University, and all experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

METHODS

Real-Time Polymerase Chain Reaction: RNA was extracted from 7-daytestes by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and RNA concentration and purity (as determined by 260/280-nm absorbance reading ratios) were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific).For each sample, 1μg of RNA was reverse transcribed with oligo(d)T priming by using a Prime Script RT Master Mix Perfect RealTime Kit (TAKARA). The cDNA was amplified with the SYBR Premix Ex Taq^{TM}II kit (TAKARA) on a LightCycler480 (Roche). *Actb* served as the reference gene, and samples without cDNA or without oligonucleotides were used as negative controls. The control *Actb* fragment was amplified using the following primer sequences: *Actb* forward, 5'-GACGGCCAGGTCATCACTAT-3'; and *Actb* reverse, 5'-ATGCCACAGGATTCCATACC-3'. The *Ngn3* primers were as follows: *Ngn3*-forward: 5'-AGTGCTCAGTTCCAATTCCAC-3'; and *Ngn3*-reverse: 5'-CGGCTTCTTCGCTTTTTGCTG-3'. The 25 μl PCR included 5 μl 10 × diluted cDNA, 12.5 μl SYBR Premix EX Taq^{TM}II,2μl of primers, and 5.5μl H_2O. The reaction was carried out for 45 cycles of 5 s at 95 °C, 20 s at 60 °C. The threshold cycle (Ct) data were determined from the default threshold settings, and all measurements were performed at least three times.

Western blot analysis: 7-day testis total protein was extracted through the use of Protein Extraction Kit (Thermo, USA), then 30 μg of total cell extract was separated on SDS-polyacrylamide gel, transferred to a PVDF membrane (Millipore, Tokyo, Japan), and incubated overnight at 4 °C with the specific antibody (NGN3, 1:1000; Abcam; GAPDH, 1:4000; Santa Cruz). Bound primary antibodies were detected with peroxidase-coupled anti-mouse or anti-rabbit secondary antibodies, and immunoreactivity was visualized by chemiluminescence (Western Lightning, PerkinElmer Life Sciences, Japan) according to the manufacturer's instructions. Digital images were captured after 30 sec of exposure by using a ChemiDoc XRS molecular imaging system (Bio-Rad Laboratories).Densitometric analysis of bands was conducted using Quantity One software (Bio-Rad Laboratories), and expression levels of NGN3 protein were compared between samples based on the normalized ratio of NGN3 to GAPDH.

Chromatin immune precipitation: Testes of 7-day mice were used for Chromatin immunoprecipitation (ChIP).After tearing, tissues were fixed with 1% formaldehyde in PBS for 10 min at 37°C, to cross-link protein-DNA complexes. After nuclear protein extraction, thesamples were sonicated to fragment genomic DNA to the size ~1 kb.Then ChIP assay was performed with an EZ-ChIP™ - Chromatin Immunoprecipitation Kit (Millipore) according to the manufacturer's instructions. Protein-DNA complexes were immune precipitated overnight in the presence of the specific anti-NGN3 antibody (Abcam) or anti-SOHLH1 antibody (Abcam). Purified DNA was used for PCR analysis. Genomic regions of *Sohlh1* or *Ngn3*promoter containing the E-boxes binding sites were amplified by PCR using specific primers : *Sohlh1*-E-box F: 5'-ACTAGAGACGGGTTACTT-3'*Sohlh1*-E-box R: 5'- TGGAGGGAAGCAACAAGC-3'; *Ngn3*-E-box F: 5'-CCCCAAACCTCCTTCATG-3', *Ngn3*-E-box R: 5'-CCTGGCACGCTTTATCTG-3'. Primer efficiency was examined by genomic DNA before ChIP analysis.

***Ngn3* expression vector construction:** The gene encoding mouse *Ngn3*was amplified from cDNA of mouse testis. Specific primers were: *Ngn3* CDS-F: 5'-ATGGCGCCTCATCCCTTG-3', *Ngn3* CDS-R: 5'-CAAGAAGTCTGAGAACACCA-3'. The PCR products were ligated into pUCm-T vector (Beyotime, China) first, and then subcloned into pEGFP-N1 with BglII and KpnI. The final expression vector was sequenced to verify construction.

Mouse *Sohoh1*promoter luciferase reporter constructs: We analysed the upstream region of *Sohlh1*(GenBank Gene ID: 227631) –2000 bp to +500 bp using Berkeley Drosophila Genome Project Neural Network Promoter Prediction software (http:// www.fruitfly.org/seq_tools/promoter.html), and designed seven pairs of PCR primers based on the results (shown in Table 1):

Table 1. PCR primers for construction of *Sohlh1*promoter Luc plasmid

Primer name	Sequence	Product size
Sohlh1-A Luc	F: 5'-CGGGGTACC AGCATTCAAGTCTACGAGCCTAT-3' R: 5'- GGAAGATCT TGGAGGGAAGCAACAAGC-3'	2399bp (-2357~+42)
Sohlh1-B Luc	F: 5'-CGGGGTACC GGAGGTGGCCTTGTTGTGT-3' R: 5'- GGAAGATCT TGGAGGGAAGCAACAAGC-3'	2069bp (-2027~+42)
Sohlh1-C Luc	F: 5'-CGGGGTACCTAAATGAGTATATGGCGAC-3' R: 5'- GGAAGATCT TGGAGGGAAGCAACAAGC-3'	1685bp (-1644~+42)
Sohlh1-D Luc	F: 5'-CGGGGTACCCCCTATCCTTAGTCAAAGGC-3' R: 5'- GGAAGATCT TGGAGGGAAGCAACAAGC-3'	1346bp (-1304~+42)
Sohlh1-E Luc	F: 5'-CGGGGTACCTGTATCATTTCCTCTGTGGC-3' R: 5'- GGAAGATCT TGGAGGGAAGCAACAAGC-3'	1034bp (-993~+42)
Sohlh1-F Luc	F: 5'-CGGGGTACCGAGTGTTTCACCAGCGAAGC-3' R: 5'- GGAAGATCT TGGAGGGAAGCAACAAGC-3'	694bp (-652~+42)
Sohlh1-G Luc	F: 5'-CGGGGTACCACTAGAGACGGGTTACTT-3' R: 5'- GGAAGATCT TGGAGGGAAGCAACAAGC-3'	345bp (-304~+42)

The KpnI and BglII digested sequences (underlined in Table 1) were designed in the primers, and the PCR products were subcloned into pGL3-basic with KpnI and BglII. All constructs were confirmed by sequencing.

Cell culture, transfection and luciferase assays: The ability of *Ngn3*binding the *Sohlh1*promoter region and regulate transcription was assessed using a dual firefly and Renilla luciferase assay. HEK293T cells were grown in DMEM containing 10% heat inactivated fetal bovine serum (Gibco, Carlsbad, CA, USA), at 37°C in 5% CO_2. Cells were plated on 96 well plates prior to performing transient transfection.24 h after plating, HEK293T cells were cotransfected with 100 ng of*Ngn3* expression vector and pGL3-basic vector containing a 2399-bp portion of the *Sohlh1*promoter region upstream of the firefly luciferase gene (pGL3-*sohlh1*A Luc) plasmid, using Lipofectamine-2000 reagent (Invitrogen) in Opti-MEM(Minimum Essential Media) (Invitrogen). The pEGFP-N1, pGL-3 basic, and 20ng of Renilla luciferase plasmid (pRL-TKVector; Promega) were used as control. After 4 h, Opti-MEM was replaced by DMEM supplemented with a 1% penicillin-streptomycin solution (Invitrogen) and a 1% fetal bovine serum (Invitrogen).48 h after transfection, cells were lysed by 1× passive lysis buffer (Promega) and luciferase activity was assayed with the dualluciferase assay system kit (Promega) according to the manufacturer's instructions (Promega, Madison, WI, USA). The ratio of reporter activity (firefly) to internal control activity (Renilla) was used to determine a relative luciferase unit (RLU) for comparison between treatments. Each transfection was performed in triplicate and experiments were carried out a total of three times. Different lengths of *Sohlh1*promoter region were subcloned into pGL3-basic vector as shown above, and they were cotransfected with *Ngn3* expression vector to determine the core region of *Sohlh1*promoter. The luciferase assays were performed as above.

Immunoprecipitation: The immunoprecipitation (IP) assays were performed using *Ngn3* and *Sohlh1* antibodies in wild type and *Sohlh1*KO testes. 7weekold male mice were used in immunoprecipitation for they were adult. The testes were lysed in modified RIPA buffer (1% NP-40, 0.25% deoxycholate, protease inhibitor cocktail) after sonic. Prior to IP, the lysates were precleared by incubating with Protein A or G agarose beads (Beyotime, China) for 2h at 4°C. To immune precipitate the protein of interest, the total protein was incubated with the antibody-conjugated beads overnight at 4°C. The Beads were washed twice in IP lysis buffer and once in PBS and boiled in 2×loading buffer (0.1M Tris-HCl, pH7.4, 50% Glycerol, 10% SDS, 5% 2-Mercaptoethanol, 0.5% Bromophenol Blue) before SDS-PAGE.

Statistical analysis: All experimental data were analysed using the SPSS 17.0 software (SPSS, Chicago, IL, USA). The differences in the mean value between groups were compared using one-way analysis of variance. Paired comparisons of the mean value between groups were performed using a two-sample *t*-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Expression of *Ngn3* decreased in *Sohlh1*$^{-/-}$mice: (Ballow *et al*., 2006)compared gene expression in *Sohlh1*$^{-/-}$ and wild type mice with Affymetrix 430 2.0 microarray chip, in which the microarray chip result showed that *Ngn3* is significantly down-regulated in *Sohlh1*$^{-/-}$ testes. Firstly, we use real-time PCR and western blot to confirm the microarray result. Real-time PCR showedthat the *Ngn3* mRNA was decreased in *Sohlh1*$^{-/-}$ mice compared to wild type C57BL/6 miceand

western blot showed that the protein of *Ngn3* was also decreased in *Sohlh1*$^{-/-}$ mice, which is consistent with the real-time PCR data (Figure 1).

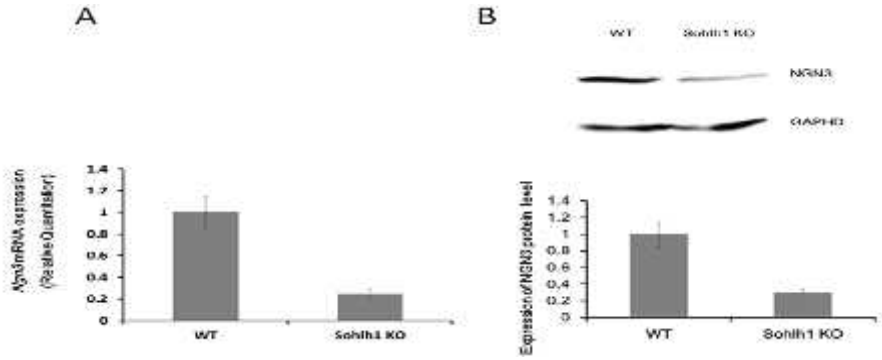

Figure 1: Expression of *Ngn3* in WT and *Sohlh1*$^{-/-}$mice. (A): Real-time PCR results showed decreased mRNA level of *Ngn3*; (B): Western blot results of *Ngn3*protein level in WT and *Sohlh1*$^{-/-}$mice.

Ngn3*bind to the promoter region of *Sohlh1: The expression of *Ngn3* decreased in *Sohlh1* knockout mice, which would be usually thought that *Sohlh1* regulates the expression of *Ngn3*. Therefore, we used*Sohlh1*antibody to perform ChIP, but the result showed that *Sohlh1* did not bind to the promoter region of *Ngn3*. The cells expressing *Ngn3* were in the prior stage of the ones expressing *Sohlh1*, thus *Ngn3* may lie in upstream of *Sohlh1*, and regulate the expression of *Sohlh1*. Then ChIP was performed with rabbit anti mouse *Ngn3*antibody. 7-day mouse testes were used in Chromatin immunoprecipitation for their high rate of spermatogonia. The DNA sequences of *Sohlh1*promoter(-2500~+500) were analyzed by a Neural Network Promoter Prediction software(a product of Berkeley Drosophila Genome Project, http://www.fruitfly.org/seq_tools/promoter.html). The prediction was showed in Figure 2A, in which the most possible start site of transcription is T (shown in larger font), -55bp upstream of *sohlh1* gene. DNA collected by ChIP was further analysed by PCR. The specific primers used for ChIP analysis covered the -304~+42 region of *Sohlh1* promoter, and there were 5 E-boxes in this area (Figure2B). The ChIP result indicated that *Ngn3*bind to the promoter region of *Sohlh1*in vivo (Figure 2C).

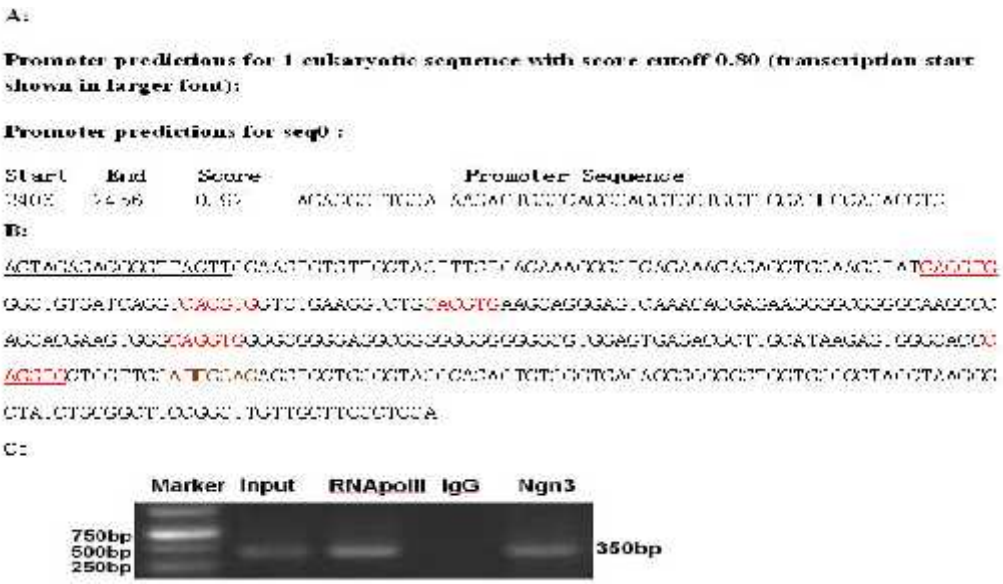

Figure2: *Ngn3*bind to *Sohlh1*promoter.(A): Prediction of *Sohlh1* transcription. Transcription start site was analyzed by software, most possible start site of transcription is T (shown in larger font), -55bp upstream of sohlh1 gene.(B): E-box in -304~+42 regionof *Sohlh1* promoter. The specific primers designed for ChIP analysis cover the -304~+42 region of *Sohlh1*promoter, and there were 5 E-boxes (CANNTG, shown in red and with underline) in this area. *Ngn3*may bind one of the five E-boxes.(C):ChIP result with *Ngn3*antibody.*Ngn3*protein could bind to the promoter region of *Sohlh1*.

***Ngn3*increased the transcription of *Sohlh1*in vitro:** To determine whether *Ngn3*could activate the *Sohlh1* promoter, we performed reporter gene assay using HEK293T cell line for its high transfection efficiency. The -2357~+42 region of *Sohlh1*promoter was subcloned into pGL3-basic vector (named *Sohlh1*-A-Luc). The *Ngn3* expression vector and *Sohlh1*-A-Luc vector were cotrasfected into HEK293T cells. As shown in Figure 3, *Ngn3*enhanced the *Sohlh1* reporter activity in HEK293T cells. These data suggest that *Ngn3* could increase the expression of *Sohlh1in vitro*.

To further define the function of *Ngn3* in the activation of the *Sohlh1* promoter, we generated several deletion promoter constructs (A, B, C, D, E, F, and G) in the pGL3 luciferase reporter vector and performed reporter analysis in HEK293T cells. The *Sohlh1*-A to G promoter, were all activated by co-expression of *Ngn3*. There is no difference between the 7 promoters. These results suggest that -304~+42 promoter region of *Sohlh1*may be critical for*Ngn3* binding.

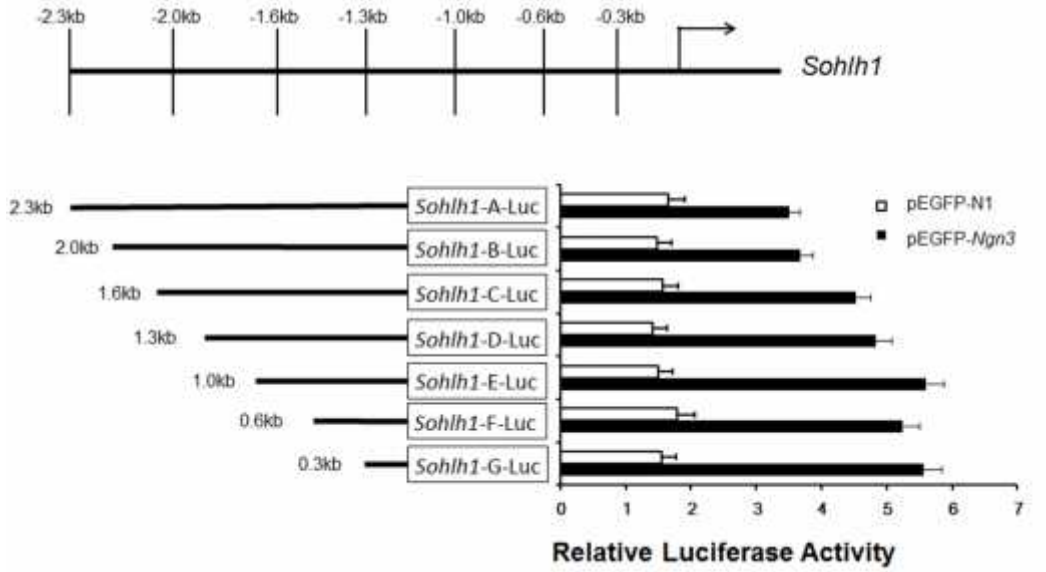

Figure 3: Results of luciferase assays. pEGFP-*Ngn3* plasmid and different length of pGL3-*Sohlh1*-basic plasmids were co-transfected into HEK293T cells. Compared to pEGFP-N1, pEGFP-*Ngn3* could increase the expression of *Sohlh1in vitro*.

Interaction of NGN3 protein andSOHLH1 in testis: Since we revealed that *Ngn3* could bind to the promoter of *Sohlh1*and could promote the transcription of *Sohlh1* in vitro, we then take a step forward to perform IP to identify whether NGN3 could bind withSOHLH1 protein to regulates permatognesis in testis. As shown in Figure 4, SOHLH1 was detected in NGN3 complexes immune precipitated from 7week-old mice testis extracts, and NGN3 was also confirmed in the reciprocal SOHLH1 immunoprecipitation. We also performed the same experiments using *Sohlh1*$^{-/-}$ testes, but no IP bands were detected (data not shown). Taken together, our data indicate that NGN3andSOHLH1 protein formed a complex, which may function together.

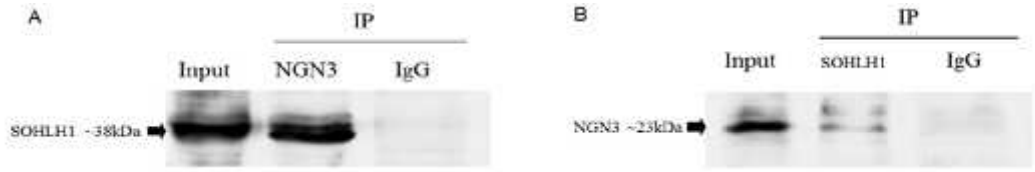

Figure 4: Interaction of NGN3 and SOHLH1 in WT testis.(A) : Co-immunoprecipitation (IP) of SOHLH1 with anti-NGN3 antibody. (B): The reciprocal co-immunoprecipitationwith anti- SOHLH1 antibody.

DISCUSSION

Spermatogenesis is a complex process that generates millions of spermatozoa per day in mammals, in which SSCs play a key role and can self-renew to maintain the amount of spermatogonia and can differentiate into spermatogonial progenitors(Song and Wilkinson, 2014). Several transcription factors have been

identified that can promotes permatogonial differentiation (DMRT1, NGN3, SOHLH1, SOHLH2, SOX3, and STAT3), some of which may affect the fate of SSCs either to differentiate or to promote later spermatogonial differentiation steps. Many of these transcription factors regulate each other and act on common targets, suggesting they integrate to form complex transcriptional networks in self-renewing and differentiating spermatogonia. In this study, we identified that *Ngn3*could bind to the promoter of *Sohlh1*, and promote the transcription of *Sohlh1*.

Ngn3, recognized for its role in promoting the development of cells in pancreas and(Gu *et al*., 2002; Heremans *et al*., 2002) neurons in the hypothalamus (Pelling *et al*., 2011; Simon-Areces *et al*., 2011). In testis, *Ngn3*expressed in a sub-set of undifferentiated spermatogonia (Yoshida *et al*., 2004), was thought to be one of the transcription factors that promote the conversion of spermatogonial progenitors into differentiating A-spermatogonia. *Ngn3*-positive spermatogonia distributed at a low frequency throughout the cell cycle and with the highest frequency at stages VII–VIII(Yoshida *et al*., 2004). This is compatible to the distribution of As, Apr, and Aalspermatogonia, which transform into A1 spermatogonia at seminiferous tubule stages VII–VIII(Chiarini-Garcia and Russell, 2001; Tegelenbosch and de Rooij, 1993). Kaucher *et al*., (2012) found that depletion of *Ngn3*led to an increase in the number of SSCs without measurably increasing cell proliferation, but by the blockade of SSC differentiation. However, the molecular circuitry downstream of *Ngn3* that drives SSC differentiation is unknown. As a member of the bHLH family, *Ngn3*could bind E box elements within promoters of target genes to regulate transcription (Davis *et al*., 1990; Murre *et al*., 1989). Studies of the pancreatic endocrine celllineage revealed a variety of genes regulated by*Ngn3*, including neurogenic differentiation 1 (*Neurod1*) (Gradwohl *et al*., 2000; Heremans *et al*., 2002), transcription factor LIM/homeodomain (*Isl1*) (Gradwohl *et al*., 2000), paired box gene 4 (*Pax4*)(Gradwohl *et al*., 2000; Heremans *et al*., 2002), and paired box gene 6 (*Pax6*) (Gradwohl *et al*., 2000; Heremans *et al*., 2002). Scanning of microarray gene expression database for mouse undifferentiated spermatogonia indicates that these genes above are not expressed (Oatley *et al*., 2007). Thus, the majority of downstream targets of *Ngn3* for transcriptional regulation in the male germline are either distinct from those in pancreatic precursors or express later in germ cell development when transition to differentiating spermatogonia occurs.

*Sohlh1*seems to be a possible downstream target of *Ngn3*.In males, distribution of SOHLH1 protein in the mouse seminiferous epithelium of adult testes was analyzed by immunohistochemistry. SOHLH1 is initially detected in Stage IV Aalspermatogonia and strongly expressed in Aal, A1, A2, A3, A4, Intermediate and Type B spermatogonia. Both *Ngn3* and *Sohlh1* were expressed in Aalspermatogonia, which was the key point of undifferentiated spermatogonia transition to differentiated spermatogonia. Loss of *Sohlh1*, undifferentiated spermatogonia failed in differentiating to spermatocytes.*Sohlh1* directly stimulates *Kit* transcription in postnatal spermatogonia, thus activates the signalling involved in spermatogonia differentiation and spermatogenetic progression(Barrios *et al*., 2012).

To summarize, we propose that *Ngn3* and *Sohlh1*may interact and regulate spermatogenesis for the following three reasons: i, cells which expressing *Ngn3* is overlapped with cells expressing *Sohlh1*;ii, both *Ngn3* and *Sohlh1* regulate the differentiation of spermatogonia; iii, microarray array(Ballow *et al*., 2006) and our real-time PCR, western blot results show that *Ngn3* expression decreased while *Sohlh1* is absent. Our data strongly indicated there are associations between *Ngn3* and *Sohlh1*.We used 7-day wild type and *Sohlh1*$^{-/-}$ mice in these experiments to evaluate the expression level of *Ngn3*, in which most cells in 7-dayseminiferous tubules were spermatogonia that haven't yet differentiated into spermatocytes and 7-d *Sohlh1*$^{-/-}$testes were similar to wild type testis in histology, which indicated that the amount of spermatogonia in *Sohlh1*$^{-/-}$ testis was approximately equal to wild type testis. Thus, we think the decrease of *Ngn3* in testis is due to the reduced expression, but not the amount of spermatogonia.

We analysed the relationship between *Ngn3*and *Sohlh1* by ChIP, the results show that *Ngn3*could bind to the promoter of *Sohlh1*;*Ngn3* may regulate the transcription of *Sohlh1*. To determine whether *Ngn3* could activate *Sohlh1* transcription, we performed luciferase reporter gene assay. We co-transfected *Ngn3* expressing vector and *Sohlh1* promoter luciferase reporter vectors into HEK293T cells, in contrast to pEGFP-N1 plasmid, the *Ngn3* expressing vector increase the luciferase of *Sohlh1* promoters. It indicated the protein of *Ngn3*could promote the transcription of *Sohlh1*. Forther more, the co-IP results show the protein interaction of NGN3 and SOHLH1. Before our report, there is only one paper about the relation between *Ngn3* and *Sohlh1*, it is written by Ballow *et al*., (2006) in 2006, in their study the microarray array result show that the *Ngn3* decrease in *Sohlh1*$^{-/-}$ mouse. Then some articlescited this paper, guessing*Ngn3* may be regulated by *Sohlh1*, but there is no other experiment result supporting that idea. Our study showed the transcription regulation relationship between *Ngn3* and *Sohlh1* by ChIP and luciferase reporter gene assay for the first time, and proved that NGN3 protein may regulate spermatogenesis by forming complex with SOHLH1.Since Kaucher *et al*. (2012) reported that *GDNF* could negatively regulate *Ngn3* by *STAT3*; and Barrios *et al*.(Barrios *et al*., 2012)identified that Kit was the target of *Sohlh1* during spermatogenesis; Together

with our results, it could demonstrate that *GDNF* regulate SSC differentiation through *STAT3-Ngn3-Sohlh1-Kit* pathway. Our study shows that *Sohlh1* is the direct target of *Ngn3* in differentiation of SSC.

Our results support the idea that *Ngn3* is a key role in differentiation of SSC, and it is consistent with the role of *Ngn3* in differentiation of pancreatic precursors. In the developing pancreas, *Nng3* functions as a pro-endocrine factor, which is sufficient to force undifferentiated pancreatic epithelial cells to become islet cells (Gu *et al*., 2002). But the signal pathway may be different in different organs, as mentioned above, the targets of *Ngn3* in pancreas, such as *Neurod1, Isl1, Pax4, Pax6*, were not expressed in testis; and it has been report that *GDNF* enhances *Ngn3* gene expression and beta-cell proliferation in the developing mouse pancreas (Mwangi *et al*., 2010), while *GDNF* inhibits *Ngn3* in testis(Kaucher *et al*., 2012).

Matson *et al.*(2010) reported *DMRT1*promotes spermatogonial differentiation, and the downstream target is *Sohlh1*. Chromatin immunoprecipitation studies show DMRT1 binding to the *Sohlh1* promoter. The bound region shares similarity to the DMRT1 known DNA binding consensus site. Moreover, *Dmrt1* is unlikely to be the only regulatorof*Sohlh1*, as *Dmrt1* conditional knockouts retain KIT expression and complete spermatogenesis. *Sohlh1* mutants do not express KIT in adult spermatogonia, and meiotic-like cells eventually die. It is therefore likely that a sufficient quantity of SOHLH1 proteins remain in the *Dmrt1* mutant(Murphy *et al*., 2010). Our result raised another direct regulator of *Sohlh1*, which may be the main regulator in differentiation of SSC.Recently,*Ngn3* was reported function in promoting meiosis by up-regulating *Stra8*, an important gene in spermatogenesis(Tang *et al*., 2014). SOHLH1 and SOHLH2 could heterodimerize with each other in vivo, as well as homodimerize. SOHLH proteins affect spermatogonial development by directly regulating *Gfra1, Sox3* and *Kit* gene expression. SOHLH1 and SOHLH2 suppress genes involved in SSC maintenance, and induce genes important for spermatogonial differentiation (Suzuki *et al*., 2012).It is possible that many spermatogonial differentiation transcription factors regulate each other, and there may be a large and complex gene net of the transcription factors that promote SSCs and spermatogonial differentiation, even associated with self-renew of SSCs. In the future, further studies will be performed to understand the regulation net above.

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