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NOBOX 基因的 cDNA 克隆及其特性分析

李贵强, 李美丽, 方威, 王伟, 贺彧, 宋晓光, 徐银学

南京农业大学动物科技学院, 南京 210095

摘　要: *NOBOX*(新生儿卵巢同源基因)是一个卵母细胞特异性表达的同源基因, 在早期滤泡发生中起重要的作用。本研究结合电子克隆的方法, 从猪卵母细胞中成功地克隆了 *NOBOX* 基因的全长 cDNA 序列(GenBank Accession No. FJ587509)。猪 *NOBOX* 基因的 cDNA 全长为 1768 bp, 包含 1419 bp 的开放阅读框。生物信息学分析表明 *NOBOX* 基因编码了 472 个氨基酸, 分子量为 51.08 kD, 等电点为 5.73。该蛋白定位于细胞核中, 含有一个保守的结构域——cd00086。借助 Clustalw 软件, 采用 N-J 算法构建了 NOBOX 蛋白的系统进化树, 分析了不同物种间的进化关系。应用实时荧光定量 PCR 技术分析该基因在母猪不同组织、细胞及 4 种孤雌激活胚胎的表达模式, 结果表明该基因在母猪各组织中均有不同程度的表达, 其中在心脏、肾脏和卵母细胞中表达水平较高, 推测其可能在心脏、肾脏和卵母细胞中发挥着重要的作用; NOBOX 基因在胚胎发育阶段的表达水平高于 G-V 期的卵母细胞, 表明在胚胎发育阶段 pNOBOX 的表达增强。

关键词: 猪, NOBOX 基因, cDNA 克隆, 生物信息学分析, 基因表达

Cloning and characterization of porcine *NOBOX* gene

Guiqiang Li, Meili Li, Wei Fang, Wei Wang, Yu He, Xiaoguang Song, and Yinxue Xu

College of Animal Science and Technology, *Nanjing Agricultural University*, *Nanjing* 210095, *China*

Abstract: Newborn ovary homeobox gene (*NOBOX*) is an oocyte-specific homeobox gene that plays a critical role in early folliculogenesis and represents a candidate gene for nonsyndromic ovarian failure. We used *in silico* approach in combination with rapid amplification of cDNA ends (RACE) to clone the full-length cDNA of *NOBOX* (GenBank Accession No. FJ587509) from porcine oocytes. It contains 1768 bp nucleotides, with an open reading frame (ORF) of 1419 bp. The putative porcine NOBOX gene encodes 472 amino acids with the molecular weight of 51.08 kD and pI of 5.73. Bioinformatics prediction indicates that this protein contains a cd00086 homeodomain. Real-time PCR analysis showed that the *NOBOX* gene is expressed in various tissues, oocytes and embryos cells (4-cell, 8-cell, morula and blastocyst) at different expression levels. The expression levels of this gene in heart, kidney and oocytes are higher than that in other tissues, which suggested that the NOBOX protein might play an important role in those tissues. The expression of *NOBOX* in developmental stages is higher than that in GV-stage oocytes, which suggested that the expression of *pNOBOX* was enhanced in developmental stages.

Keywords: pig, *NOBOX* gene, cDNA cloning, bioinformatics analysis, gene expression

Introduction

Folliculogenesis in mammals is a complex process requiring both extraovarian and intraovarian factors. Follicular growth depends upon both oocyte and surrounding somatic cells (i.e. granulosa and theca

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Corresponding author: Yinxue Xu. Tel: +86-25-84395278; Fax: +86-25-84395314. E-mail: xuyinxue@njau.edu.cn
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cells). Within the mammalian ovary, oocytes play an important role in regulating folliculogenesis. Growth differentiation factor 9 (*Gdf9*)[1], bone morphogenetic protein 15 (*Bmp15*)[2], Pou5f1 (POU domain, class 5, transcription factor 1; also known as *Oct4*)[3], zona pellucida genes (*Zp1, Zp2* and *Zp3*)[4], factor in the germline alpha (*Figla*)[5], sal-like 4 (*Sall4*)[6], the c-mos protooncogene (*c-mos*)[7], and newborn ovary homeobox-encoding gene (*NOBOX*)[8] are some of the genes preferentially expressed in oocytes which play important roles during folliculogenesis. Recently, scientists pay more attention to *NOBOX* gene. The murine *NOBOX* gene was identified by *in silico* cDNA library subtraction as an unique expressed sequence tag residing in a mouse newborn ovary cDNA library[8]. The gene which has been mapped to chromosome 6 spans 14 kb and is encoded by eight exons in mice. The corresponding region that is on human chromosome 7q35 has been identified as a syntenic region on murine chromosome 6[9].

Lack of *NOBOX* accelerates loss of postnatal oocyte and abolishes the transition from primordial to growing follicles in mice. And the follicles would be replaced by fibrous tissue in female mice in *NOBOX* $^{-/-}$ females[10]. When these females have normal ovaries at birth, oocyte growth beyond the primordial stage is inhibited, leading to wide-scale loss of oocytes in 14 days. Several genes preferentially expressed in oocytes, such as *Gdf9, Pou5f1,* and *Mos*, are drastically down-regulated in ovaries deficient in *NOBOX*[11].

In addition, further study identified several *cis-acting* sites, TAATTG, TAGTTG, and TAATTA as *NOBOX* DNA Binding Elements (NBEs) using a library of randomly generated oligonucleotides by cyclic amplification of sequence target assay and mutation analyses. *NOBOX* preferentially binds to the NBEs with high affinity and directly regulates the transcription of *Pou5f1* and *Gdf9* in oocytes during early folliculogenesis[12].

Although the *NOBOX* gene has been identified in mice and humans, its physiological function has not been fully investigated. Moreover, in this report, we cloned the full-length cDNA of porcine *NOBOX* using the *in silico* approach and RACE, and analyzed its physiological function using the basic bioinformatics method. Real-time fluorescence quantitative PCR was conducted to analyze the relative expression levels in various tissues, oocytes and cells of developing embryos. This study provides primary information for further understanding to the biochemical function of *NOBOX gene* in pigs.

1 Materials and methods

1.1 Tissue samples treatment

One male and one female, 40-day old Erhualian pigs (one of the strains of Taihu pig breed) were slaughtered for sampling. Fresh porcine tissues (testis, brain, ovary, liver, heart, lung, thymus, spleen, kidney, and skeletal muscle) were instantly frozen in liquid nitrogen and stored at –80 °C for use.

1.2 Oocytes collection, electrical activation, and *in vitro* culture (IVC)

Porcine ovaries were obtained from Landrace gilts at a local slaughterhouse and transported to the laboratory within 2 h in a thermos flask with 35 °C–37 °C sterile physiological saline. Ovaries were washed twice with 37 °C sterile physiological saline containing 100 IU/L penicillin and 50 mg/L streptomycin. The cumulus–oocyte complexes (COCs) were aspirated from follicles (2–6 mm in diameter) with an 18-gauge needle attached to a disposable 10 mL syringe. After being washed three times with Tyrode's lactate (TL)-Hepes-PVA (polyvinyl alcohol, 0.1%)[13], oocytes with uniform ooplasm and compact cumulus cells were selected for use. From obtained above oocytes, some COCs were digested in 0.1% hyaluronidase at 39 °C for 5 min, and then the cumulus cells were stripped off by gentle pipetting to obtain denuded oocytes (DOs). Some COCs and denuded oocytes were cultured in maturation medium respectively as groups of COCs and DOs; the other COCs and denuded oocytes were used in total RNA extraction directly. A total of 20–30 COCs or DOs were transferred into a 100 μL microdrop of maturation medium, and all groups were cultured in a 39 °C humidified incubator containing 5% CO_2 in air for 44 h[14]. The maturation medium consisted of TCM199 (Sigma, USA) supplemented with 10% porcine follicular fluid (PFF), 0.1 mg/mL cysteine, 0.065 mg/mL penicillin, 10 ng/mL epidermal growth factor (EGF), 10 IU/mL equine chorionic gonadotropin (eCG; Biosis, Shanghai), and 10 IU/mL human chorionic gonadotrophin (hCG; Biosis, Shanghai). Then, morphologically mature oocytes were washed twice, equilibrated in activation medium for 30–60 s and exposed to one DC electrical pulse (120 V/mm for 80 μs). The medium contained 0.3 mol/L mannitol, 0.05 mmol/L $CaCl_2$, 0.1 mmol/L $MgCl_2$, and 0.1% BSA. The parthenogenetically activated (PA)

oocytes were initially cultured in NCSU-23 supplemented with 2 mmol/L 6-DAMP for 6 h, and afterwards added in droplets of NCSU-23 medium containing 0.4% BSA (15–20 embryos/50 μL) at 39 °C, in an atmosphere of 5% CO_2 in humidified air for 8 d. The embryos including 4-cell, 8-cell, morula and blastocyst were chosen for RNA extraction.

1.3 Total RNA extraction and reverse transcription

The tissues and pooled oocytes (one pool represent about 50 porcine oocytes) were used for isolating total RNA using the Trizol reagent (Invitrogen, California, US) according to the manufacture's instructions. The yield and quality of total RNA were determined spectrophotometrically using 260 nm/280 nm absorbance ratio using an UV-754 photometer (SMOIF, Shanghai, China).

Total RNA from each pool of cells and other tissues was independently transcribed to cDNA using M-MLV reverse transcriptase (Promega, Madision, USA).

1.4 Rapid amplification of cDNA ends (RACE)

Several EST sequences of porcine *NOBOX* gene were found via BLAST program in the Trace/*Sus_scrofa* WGS database and the Trace/*Sus_scrofa* EST database in NCBI (National Center for Biotechnology Information) respectively. Two of them, EST1 (gnl|ti|470952394) and EST2 (gnl|ti|857218241), were chosen to design the gene-specific primers for 3′ RACE (Table 1). Total RNA from each pool of oocytes was transcribed to cDNA using GeneRacer™ Oligo dT primer (provided in GeneRacer™ Kit, Invitrogen, Shanghai, China). For increasing specificity and reduced background amplification, Touchdown PCR was performed as follows: denaturation at 94 °C for 3 min, followed by 5 cycles at 94 °C for 30 s, 72 °C for 3 min, 5 cycles at 94 °C for 30 s, 70 °C for 30 s and 72 °C for 3 min, 27 cycles at 94 °C for 30 s, 68 °C for 30 s and 72 °C for 3 min and a final extension of 72 °C for 7 min. Then, the products of Touchdown PCR were used as templates for nested PCR, which was performed as follows: denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 61 °C for 30 s, 72 °C for 1 min 30 s and a final extension of 72 °C for 7 min. PCR products were confirmed by agarose gel electrophoresis, gel purification and subcloning to pMD18-T Vectors (TaKaRa, Dalian, China). A positive clone was sequenced on an ABI3730 sequencer (Invitrogen, Shanghai, China).

According to the sequence obtained above, the gene-specific primers were designed (Table 1), and 5′ RACE was performed using the GeneRacer™ Kit (Invitrogen, Shanghai, China). The programs of PCR were same as described above.

1.5 Bioinformatics analysis

The protein theoretical molecular weight and isoelectric point prediction, hydropathy analysis, transmembrane region prediction, signal peptide prediction and subcellular localization prediction were performed using Compute PI/Mw (http://au.expasy.org/tools/pi_tool.html), protscale (http://us.expasy.org/cgi-bin/protscale.Pl), TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/), SignalP3.0 (http://genome.cbs.dtu.dk/services/signalP/) and the PSORT II program (http://psort.ims.u-tokyo.ac.jp/form2.html), respectively. The BLASTp program was used to search for similar proteins under the expected value of 0.0 (http://www.ncbi.nlm.nih.gov/Blast). Amino acid sequences of NOBOX of various vertebrates were collected from GenBank and aligned by computer program (ClustalX Ver.1.81). Phylogenetic tree were calculated with the same program and displayed by PhyloDraw V0.82. To create this tree, neighbor-joining (N-J) method was used. The CD-search service was used to identify the conserved domains present in a protein (http:www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

1.6 Real-time PCR analysis

Two micrograms of total RNA from each sample were reverse transcribed into cDNA with oligo-dT primers using the M-MLV reverse transcriptase (Promega, Madision, USA) according to the manufacturer′ s instructions. Primers for the real-time PCR were designed using Primer Premier5.0 and commercially synthesized (Invitrogen Ltd. Shanghai). The *β-actin* gene was amplified with primer A-For and A-Rev, whereas, the *NOBOX* gene was done with N-Q-For and N-Q-Rev (Table 1).

Each reaction was carried out thrice in a total volume of 20 μL with 2 μL of cDNA, 10 μL 2× PCR master mix (TaKaRa, Dalian, China), 1 μL primer mixture and 7 μL H_2O. Real time PCR was performed using a PTC200 device (MJ research, Ramsey, USA) with the following cycling parameters: 94 °C for 3 min followed by 40 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s followed by amplicon dissociation.

Table 1 Specific primers used in this study

Primers	Nucleotide sequences (5′–3′)	References/GenBank Accession Nos.	Purpose
N-3-For1	GCCTTGTGGGTCAGGCTGGGGGT	gnl|ti|470952394	3′ RACE
N-3-For2	CCCCAGAGGCCATCTGCCAAGGT	gnl|ti|470952394	3′ RACE
N-3-For3	GGTTCCAGAATCGCCGGGCAAAGT	gnl|ti|857218241	3′ RACE
N-5-Rev1	AGTAAGAGCAGGCGGGTGGTGGC	Sequenced nucleotide	5′ RACE
N-5-Rev2	GCCACTTTGCCCGGCGATTCT	Sequenced nucleotide	5′ RACE
N-5-Rev3	GTTCGAGTCTTTTTCCGACCTTGGC	Sequenced nucleotide	5′ RACE
N-Q-For	TCTGTTTGCTGCCCCGTAT	Sequenced nucleotide	Real-time PCR
N-Q-Rev	GGGAACCCTGACTTCTGCC	Sequenced nucleotide	Real-time PCR
A-For	GGACTTCGAGCAGGAGATGG	DQ845171	Real-time PCR
A-Rev	GCACCGTGTTGGCGTAGGG	DQ845171	Real-time PCR

2 Results

2.1 Molecular cloning of a full-length porcine cDNA homologue to *hNOBOX* from porcine oocytes

We initially hypothesized that the *NOBOX* homologue of the pig has similar expression and distribution patterns to *hNOBOX*, thus may be mainly expressed at a high level in the porcine oocytes which can be used as the source for the cloning of the unknown *pNOBOX* cDNA. A sequence similarity search from the database of the Swine Genome Sequencing Project (SGSP, http://www.ncbi.nlm.nih.gov/sites/entrez?Db=genomeprj&cmd=showDetailView&TermToSearch=13421) in NCBI did not yield any sequences of *NOBOX* homologues. In addition, other predicted *NOBOX* homologues from domestic animal species had not been released from the genome databases except *Bos Taurus* when this project was started.

We obtained two EST sequences through the BLAST program using the sequences of the human and *Bos Taurus NOBOX* gene, respectively. Based on the two EST sequences, three primers were designed to amplify the 3′-proximal region of the cDNA by 3′ RACE PCR. So specific primer N-3-For1 and GeneRace™ 3′ primer were used for Touchdown PCR. Then, Nested PCR was performed with primers N-3-For2 and GeneRace™ 3′ Nested Primer, N-3-For3 and GeneRace™ 3′ Nested Primer respectively, and approximately 1.2 kb and 1 kb fragments were obtained (Fig. 1A). Sequence analysis of the PCR product demonstrated that the 3′ full-length cDNA of porcine *NOBOX* was cloned correctly.

Based on success of 3′RACE PCR, the specific primers for 5′RACE were designed. The product of Touchdown PCR with GeneRace™ 5′Primer and primer N-5-Rev1 were used as template for nested PCR. After 35 cycles of Nested PCR amplification, approximately 750 bp (GeneRace™ 5′Nested Primer and primer N-5-Rev2) and 600 bp products (GeneRace™ 5′Nested Primer and primer N-5-Rev3) was detected (Fig. 1B). The identification of the amplified fragment was comfirmed by sequencing analysis, which proved that the full-length cDNA was cloned correctly.

A BLAST search with this cDNA did not yield any homologues sequence in pigs, indicating that it is a novel porcine equivalent of the *hNOBOX*. Hence, we

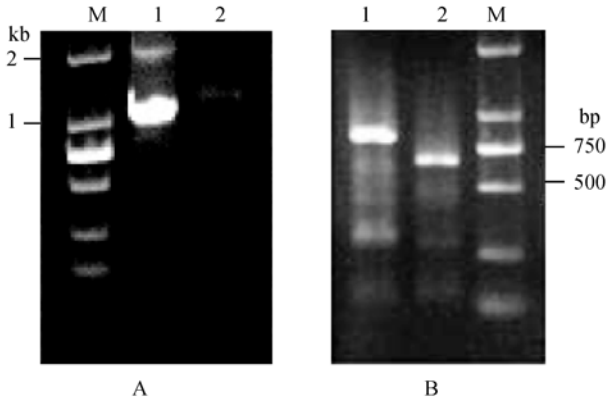

Fig. 1 Analysis of PCR products on 1% agarose gels. (A) Results of 3′ RACE PCR products. M: 2 kb DNA ladder (Bioer Teohnology, Hangzou); 1: PCR fragment of about 1 kb with N-3-For3 and GeneRace™ 3′ Nested Primer; 2: PCR fragment of about 1.2 kb with N-3-For2 and GeneRace™ 3′ Nested Primer. (B) Results of 5′RACE PCR products. M: 2 kb DNA ladder; 1: PCR fragment of about 750 bp with N-5-Rev2 and GeneRace™ 5′ Nested Primer; 2: PCR fragment of about 600 bp with N-5-Rev3 and GeneRace™ 5′ Nested Primer.

designated it as porcine *NOBOX* (*pNOBOX*). The sequence data has been submitted to the GenBank databases under Accession No. FJ587509.

2.2 Characterization of pNOBOX cDNA and its putative protein product

The 1768 bp *pNOBOX* cDNA encompassed an open reading frame (ORF) of 1419 nucleotides from 27–1445 bp. The predicted amino acid sequence has 472 residues, with a molecular weight of approximately 51.08 kD and a theoretical pI of 5.73 via the Compute PI/Mw program[15]. Hydropathy analysis showed that the NOBOX protein containes no typical hydrophobic regions by program BioAnnotator. It is predicted by SignalP3.0 that the protein has no signal peptide in its amino acids and it is a non-secretory protein.

We utilized TMHMM program to predict transmembrane region with a hidden Markov model algorithm[16], indicating that the protein was not a potential membrane protein. For subcellular localization analysis, the amino acid sequence was submitted to the PSORT II program. Using the k-nearest neighbor classification algorithm[17], the protein was presumed to resides in the nucleolus with a probability of 65.2%.

2.3 Prediction and analysis of protein structure and conserved domains

Proteins contain several modules or domains, each with a distinct evolutionary origin and function. The Conserved Domain Database alignment[18] shows that the pNOBOX protein contains a homeodomain (smart cd00086). It may be involved in the transcriptional regulation of key eukaryotic developmental processes, and may bind to DNA as monomers in a sequence–specific manner. Also, cd00086 is a member of the superfamily cl00084 (Fig. 2).

2.4 Sequence and phylogenetic analyses

In an extensive search of GenBank database for proteins with sequence similarity, the pNOBOX protein shared high identities with known NOBOX protein in other six species. The homeodomains of all seven known NOBOX proteins (*Mouse*, *Rattus norvegicus*, *Canis*, *Ornithorhynchus*, *Homo sapiens*, *Bos Taurus* and *Sus scrofa*) have highly sequence identities (Fig. 3).

The phylogenetic tree of seven NOBOX proteins was constructed based on the amino acids sequence data (Fig. 4). In this tree, the two clusters were found: one cluster consisted of *Mouse*, *Rattus norvegicus*, *Canis*, *Ornithorhynchus* and *Homo sapiens*, another consisted of *Bos Taurus* and *Sus scrofa*. These distances revealed the evolutionary relationship of various species.

2.5 Real-time PCR analysis of gene pNOBOX expression

The expression was observed in oocyte, granulocyte, ovary, testis (male), brain, spleen, lung, liver, kidney, heart, muscle and thymus. The pNOBOX transcripts were considerably detectable in oocyte, heart and kidney (Fig. 5A).

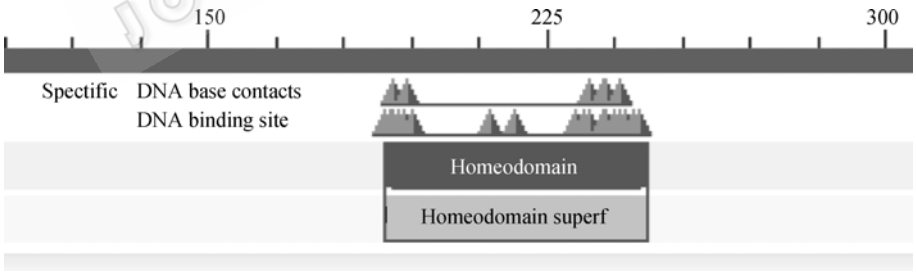

Fig. 2 Schematic representation of the porcine NOBOX protein, showing the position and type of conserved domain.

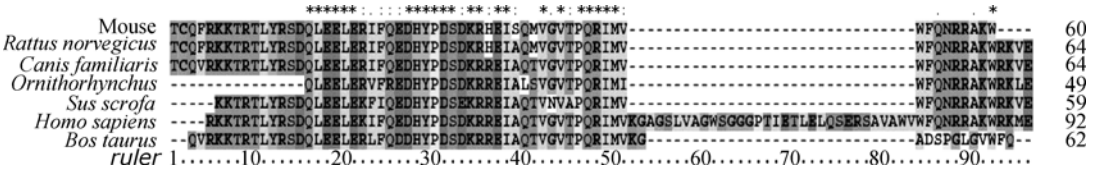

Fig. 3 NOBOX homeodomain alignment among different species. "*" marks positions with identical residues, ":" marks positions with conservative substitutions and "." marks positions with semi-conservative substitutions.

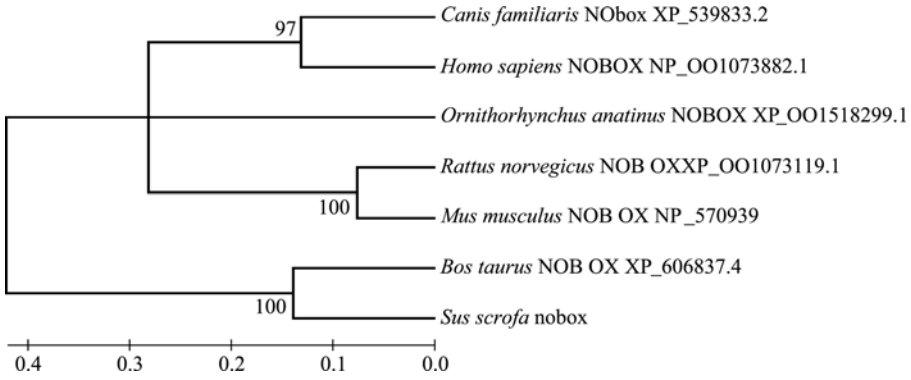

Fig. 4 Phylogenetic tree constructed by the neighbor-joining method based upon the amino acid sequences of NOBOX proteins in the seven species above. The length of branch indicates evolutionary distance.

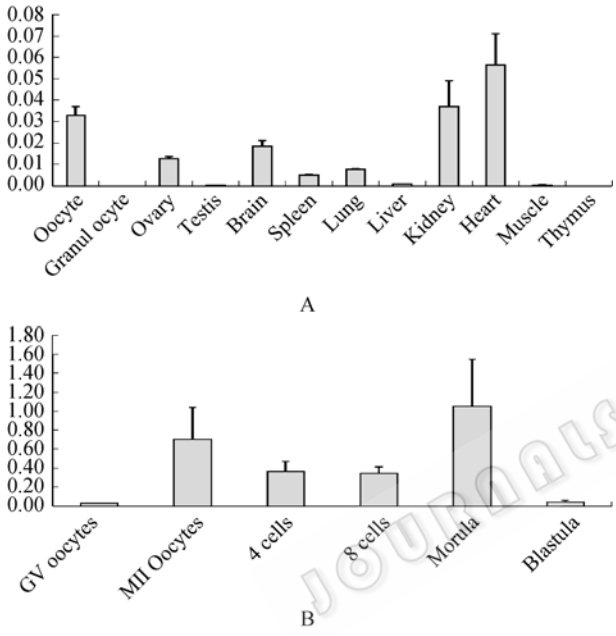

Fig. 5 Relative expression profile of *pNOBOX* mRNA analyzed by real-time PCR. The relative expression profile of pNOBOX mRNA was normalized by *β-actin* to standardise the results. *pNOBOX* was analyzed in various tissues, oocytes, and embryos cells, each sample was repeated thrice. (A) Expression levels of *pNOBOX* in oocytes, granulocytes and 10 tissues. The testis sample came from male pig, others came from female pig. (B) Expression levels in various oocytes and embryos cells.

The relative expression levels of pNOBOX mRNA were detected by real-time PCR in a different stages from GV-stage oocytes to blastocyst (Fig. 5B). The expression of pNOBOX in developmental stages (including 4-cell, 8-cell, morula and blastocyst) was higher than that in the GV-stage oocytes, suggesting that the expression of pNOBOX was enhanced in developing stages.

3 Discussion

In this study, we have cloned and characterized the full length cDNA of porcine NOBOX gene according to *in silico* cloning for the first time. Running the *in silico* cloning, the available ESTs were accumulated from the pig EST database. Due to inadequacy of the pig genome and EST data, it was difficult to obtain such a complete cDNA sequence based on the EST data, such as, human[19], mouse[20] and rice[21]. This result laid a solid foundation for further study on biological development of oocytes.

A 1768 bp full-length cDNA sequence wes finally obtained by RACE in this paper, with a 1419 bp ORF flanked by a 26 bp 5′ UTR and a 320 bp 3′ UTR. Then, basic bioinformatics analysis revealed that the porcine NOBOX mRNA encodes 472 residues with a molecular weight of 51.08 kD and a pI of 5.73. Subcellular localization analysis indicates that the protein is located in the nucleolus.

The NOBOX protein has a DNA binding domain, cd00086, which is involves in the transcriptional regulation of critical eukaryotic developmental processes, and may bind to DNA as monomers or as homo-and/or heterodimers in a sequence-specific manner. Seven of the known homeodomains share over 59% amino acid identity within the cd00086 homeodomain except *Ornithorhynchus*. cd00086 is a member of the superfamily cl00084. NOBOX homeodomain can discriminate and bind to the NOBOX DNA Binding Elements (NBEs), including TAATTG, TAGTTG, and TAATTA. During early folliculogenesis, NOBOX directly regulates the expression of oocyte-specific genes, such as *Pou5f*, *Gdf9*, *Dnmtlo* and *Zar1* by binding to these NBEs with high affinity. Disruption of the mouse NOBOX gene

causes nonsyndromic ovarian failure in females[10]. Likewise NOBOX homeobox mutation causes premature ovarian failure in human[22]. Two novel variations, p.Arg355His and p.Arg360Gln, caused missense mutations in the homeobox domain, disrupted NOBOX homeodomain binding to NBE and had a predominant negative effect on the binding of wild-type NOBOX to DNA. This result suggests that NOBOX is a critical oocyte prosurvival factor and plays a key role in early folliculogenesis.

Early study revealed that NOBOX was expressed exclusively in murine oocytes. But in our study, NOBOX transcripts were detected in many tissues (including ovary, testis, brain, spleen, lung, liver, kidney, heart, muscle and thymus), oocytes and developing embryos cells from 4-cell through to blastocyst. Using a nested PCR strategy, the expression of the human NOBOX was found only in the ovary, testis and the pancreas in the 1st round. However unclear bands were detected in many other tissues in 2nd round, but it could not be considered that NOBOX expresses in those tissues. Similar study also showed that the NOBOX expression was not observed in granulose cells[9]. This difference may due to the diversity of species. We hypothesized that the numbers of 35 cycles are excessive. A PCR was performed for 25 cycles instead of 35 cycles in our further study and the same clear bands were observed (The data not given). These evidences demonstrate that there is a discrepancy in the evolution among different mammals.

In conclusion, we isolated, characterized a critical homeobox regulator of oogenesis (NOBOX), and monitored its expression. Furthermore, we demonstrated that the expression is enhanced in developmental stages in pigs.

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