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*Original article*

# Expression analysis of multifunctional RNA-binding protein hnRNP K during development of mammalian testis

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## Abstract

Heterogeneous nuclear ribonucleoprotein K (hnRNP K), is a multifunctional protein that participates in a variety of regulatory processes of signal transduction and gene expression. To further characterize the significance of hnRNP K in different male germ cells, we investigated the expression profiles of hnRNP K at different developmental stages in pig and rat testes, and conducted a comparative analysis of expression patterns between these two species. In porcine testis development, both the mRNA and protein level of hnRNP K were down-regulated from 3 months to 8 months. However, the expression level of hnRNP K was abundant across the embryonic period in rats, and decreased gradually from 0 day post partum (dpp) to 14 dpp, then increased with the highest level presenting at 90 dpp. Immunolocalization analysis further confirmed the differential expression and localization of hnRNP K protein during testis development in pigs and rats. The results showed that hnRNP K was widely distributed in gonocytes, spermatogonia, sertoli cells and Leydig cells. The dynamic expression profile of hnRNP K may imply its crucial and potential roles in the development of the testis, which will provide a theoretical basis for the future study of molecular mechanism regulation of spermatogenesis.

**Key words:** hnRNP K, testis development, spermatogenesis, pig, rat

## Introduction

Spermatogenesis is a tightly regulated and well-studied process consisting of a series of highly specific cellular processes and multiple genes involved in the final formation of the spermatozoa (Fattahi et al. 2017). With numerous substages defined within each category, the spermatogenic cells at different stages are classified into five broad categories including gonocytes, sper-

matogonia, spermatocytes, spermatids and spermatozoa. Obviously, there are several types of somatic cells in addition to the spermatogenic cells within the seminiferous tubules and intertubular compartment, such as the Sertoli cell, Leydig cell and myoid cells, providing protection, support and nutrition for germ cells, and regulating germ cell differentiation and maturation during spermatogenesis (Berruti 1998, Bott et al. 2006, de Rooij 2009, Hai et al. 2014). Spermatogenesis

depends not only on unique cell processes, but also on a variety of factors, for instance DNA methylation, histone modification, non-coding RNA and RNA alternative splicing, which affects multiple gene and protein expression patterns (Eddy 2002, Hayashi and Saitou 2014, Robles et al. 2017).

Heterogeneous nuclear ribonucleoprotein K (hnRNP K), a multifunctional protein belonging to the hnRNP family, interacts with DNA, RNA and various proteins to participate in transcription, translation, RNA splicing, DNA repair and chromatin remodeling, and plays a key role in nervous system and ovary development as well as in the spermatogenesis and carcinogenesis process (Barboro et al. 2014, He et al. 2015, Gallardo et al. 2016, Zhang et al. 2016). The structure of hnRNP K protein mainly contains three K homology (KH) domains that can combine with DNA or RNA, and one K protein interactive region (KI) domain involved in protein interaction. It also contains a nuclear localization signal (NLS) in the N terminal and a nuclear shuttling domain (KNS) in the C terminal. In eukaryotes, hnRNP K is expressed in many types of cells and tissues, but the expression levels vary during different developmental phases. It is located predominantly in the nucleus and cytoplasm, and also in mitochondria and plasma membrane (Gallardo et al. 2016). In addition, hnRNP K is subject to several post-translational modifications, such as phosphorylation, methylation, sumoylation, ubiquitylation and acetylation, which regulate its interactions with different molecules and influence its functions. Mice with *hnRNP K* biallelic gene loss are incompatible with embryonic survival, and develop a propensity towards pleiotropic effects that impact neonatal survival and development due to haploinsufficiency, demonstrating the significant role of hnRNP K in mammalian development and survival through the regulation of cellular proliferation and differentiation (Dinh et al. 2013, Gallardo et al. 2015, Dentici et al. 2017).

Some members of the hnRNP family play key roles in the process of spermatogenesis. HnRNP L is a key regulator in the apoptosis, death and growth of spermatogenic cells, which may be involved in the process of spermatogenesis via influencing the expression of activated-caspase-3 and CEACAM1 in spermatogonia (Li et al. 2012). HnRNP H1 is one of the protective proteins inhibiting apoptosis during spermatogenesis, and may function as a direct or indirect factor together with multiple proteins in restraining the apoptosis of spermatogenic cells (Zhu et al. 2010). HnRNP G-T are efficient germ cell-specific splicing co-activators, leading to modulation of signaling pathways in testes (Liu et al. 2009). HnRNP A2/B1 are expressed during spermatogenesis from spermatogonia to round spermatids,

playing key roles in regulating testis-specific splicing events and transcriptional regulation of genes in spermatogenesis (Matsui et al. 2000, Elliott and Grellscheid 2006). However, less is known about how hnRNP K is expressed and regulated in spermatogenesis. In the present study, we firstly examined the spatial and temporal expression patterns of hnRNP K in pigs. Subsequently, rats with more developmental stages were used to validate and compare the expression profiles. Our results will lay a foundation for further study of the functions of the *hnRNP K* gene during mammalian spermatogenesis. Moreover, this study may also provide the basis for generating *hnRNP K* gene-related disease models in the future.

## Materials and Methods

### Isolation and collection of testicular tissue

Purebred large white pigs (boar) in three postnatal (3 months, 4 months and 8 months) stages were obtained from Ming Gang pig farms in Xinyang Pingqiao district. Testes samples of three different individuals were collected at each stage, and at the same time hearts, livers, spleens, lungs, kidneys, brains, fats, testes and skeletal muscles tissue samples from three individuals at 4 months were obtained to analyze the porcine tissue expression profile.

Sprague-Dawley (SD) rats were obtained from the Fourth Military Medical University (Xian, P. R. China). The animals were allowed to mate naturally. The following morning, the female rats with a vaginal plug were separated from the male mice, deemed to be 0.5 days post coitum (dpc), and the day of delivery was considered to be 0 day post partum (dpp). Testes were collected in fetal (15.5 dpc and 18.5 dpc) and postnatal (0 dpp, 7 dpp, 14 dpp, 21 dpp and 90 dpp) stages. Samples from three different individuals at each stage were fixed in carbazotic acid buffer solutions (saturation picric acid: acetic acid: methanol = 75 : 20 : 5) for 8-12 hours and the others were decapsulated and rinsed in sterile DEPC-treated saline, pooled, and frozen in liquid nitrogen for RNA extraction. Among them, 30 male rats were collected at embryonic stages, and 10 at each postnatal time point. The entire experimental procedure was approved by the Animal Care Commission of the College of Life Science, Xinyang Normal University, China.

### RNA isolation, cDNA synthesis and qRT-PCR analysis

Total-RNA extractions were carried out from different developing testes stages using TRIzol (Takara,

Table 1 Primer sequences for specific genes.

Gene	Primer sequence	Primer length	Product size	Gene Bank sequence No.
<i>P-hnRNP K</i>	F: CGCCATTATCCTCTGTTT	18 bp	126 bp	NM_001267845
	R: GTTCTCCCGAAGTTCTTT	18 bp		
<i>P-Hprt</i>	F:GTCAAGCAGCATAATCCAAAG	21 bp	134 bp	NM_001032376.2
	R:CAAGGGCATAGCCTACCACAA	21 bp		
<i>R-hnRNP K</i>	F:CGACTGCGAGCTGAGACTGT	20 bp	172 bp	XM_006253552.2
	R: ATCGGGCTTTTCTCCAATAA	20 bp		
<i>R-β-actin</i>	F:GCGTCCACCCGCGAGTACAA	20 bp	118 bp	NM_031144.3
	R:ACATGCCGGAGCCGTTGTCG	20 bp		

Japan). The first strand cDNA was synthesized by reverse transcription of 1-5 µg total RNA using M-MLV Reverse Transcriptase and Oligo (dT)<sub>15</sub> (Invitrogen, USA). qRT-PCR was performed on the ABI 7300 (ABI, USA) using the GoTaq® qPCR Master Mix (Promega, USA). Gene-specific primers were used to determine the relative expression levels of *hnRNP K* according to the standard curve method. The primer sequences are listed in Table 1. Gene expression levels were quantified relatively to the expression of the porcine *Hprt* and rat *β-actin* gene, by employing an optimized comparative Ct ( $\Delta\Delta C_t$ ) value method. The expression level was calculated as  $2^{-(\Delta\Delta C_t)}$  to compare the relative expression, and SPSS14.0 software was used for statistical analysis, one-way ANOVA was conducted to identify genes differing in expression, and  $p < 0.05$  was considered as significant.

### Western Blotting

Testes were prepared by homogenization in 0.15 M NaCl, 0.05 M Tris HCl (pH 7.5), 1 µg/µL leupeptin, 1 µg/µL aprotinin and 0.1 µg/µL PMSF. 30 µg proteins were denatured by heating at 100°C, separated by electrophoresis on 12.5% SDS-PAGE, and transferred to PVDF membranes. The primary antibodies were monoclonal mouse antibodies anti-hnRNP K (Santa Cruz, sc-28380) and anti-GAPDH (Beyotime, AG019). The membrane was incubated in the primary antibody solution (1:1000) overnight at 4°C with agitation. Anti-mouse IgG was used as secondary antibodies (Beyotime, A0286). The membrane was then incubated in the secondary antibody reagent (1:1000) for 2 hours at room temperature and washed 3 times for 5 min each time with TBS containing 0.05% Tween 20. Detection of proteins was performed using enhanced chemiluminescence (ECL). Finally, the blot was imaged using a

FluorChem M multicolor fluorescence Western blot imaging system (ProteinSimple, USA).

### Immunofluorescent assay

The cross-sections were deparaffinized in xylene for 10 min and rehydrated in a descending alcohol series. Sections were washed for 5 min in phosphate-buffered saline (PBS). Tissue sections were then performed antigen retrieval step by keeping them in boiling Tris-EDTA buffer for 10 min, and were then pretreated after being cooled to room temperature, and incubated with 5% BSA for blocking non-specific binding. The specific primary antibodies for hnRNP K were added and incubated overnight at 4°C. For the negative controls, 5% BSA was added instead of primary antibody. The next day, sections were washed 3 times with PBS after which the Cy3 labeling secondary antibody (Beyotime, A0521) was added for 2 hours at room temperature, and were then again washed 3 times with PBS. The images were visualized using a Nikon 80i with NIS-Elements software (Nikon, Japan).

## Results

### Spatial and temporal expression pattern analysis of porcine *hnRNP K* gene

To explore *hnRNP K* gene expression, total RNA was isolated from the testes of Large White pigs. qRT-PCR analysis was performed to determine the relative mRNA expression of *hnRNP K*. As shown in Fig. 1, the mRNA of *hnRNP K* was widely expressed in all the nine tissue samples obtained. The highest level of expression was observed in testes, a moderate level was detected in skeletal muscles, fats, lungs and kidneys, and a lower level was found in hearts, livers, spleens and brains. In-

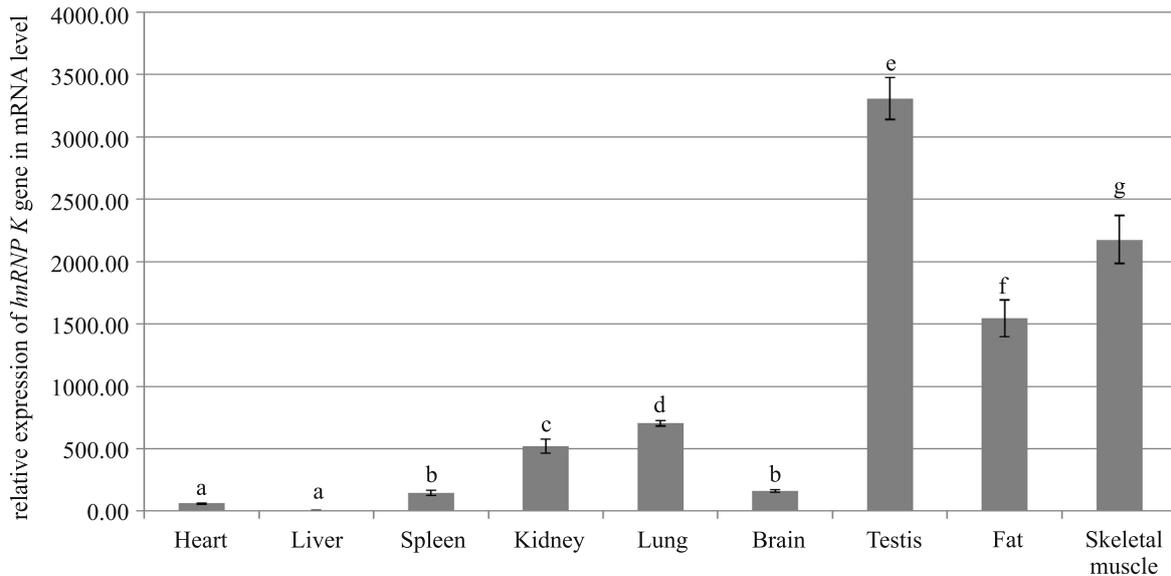


Fig. 1. Tissue distribution of porcine *hnRNP K* mRNA assessed by qRT-PCR. The values showed in this figure were the averages of three independent experiments. Error bars represented the standard deviation ( $n=3$ ) of relative mRNA expression levels of *hnRNP K* normalized to *Hprt*. Significant differences ( $p<0.05$ ) between different letters.

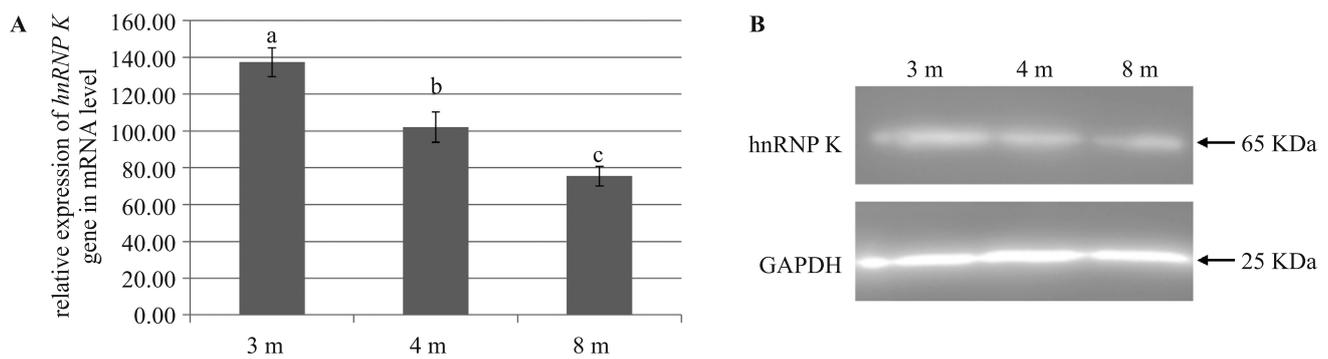


Fig. 2. Expression pattern of *hnRNP K* during porcine testis development. A: mRNA expression was analyzed by qRT-PCR, normalized to *Hprt*. B: Protein expression was analyzed by western blotting, and GAPDH expression levels were used as controls. Months (m).

terestingly, the *hnRNP K* mRNA expression level in testes was significantly higher than those for the other eight tissues ( $p<0.05$ ).

The highest expression of *hnRNP K* detected in the porcine testis may imply its potential key role in the testis. Therefore, we further examined the dynamic gene expression pattern of *hnRNP K* in the postnatal testes (3 months, 4 months and 8 months) using qRT-PCR and Western blotting. Our results revealed that the *hnRNP K* mRNA levels dropped gradually from 3 months to 8 months, and the expression level in 3 months was significantly higher than the other two stages (Fig. 2A,  $p<0.05$ ). When protein expression of *hnRNP K* was probed and compared to that of mRNA, a similar expression pattern was detected (Fig. 2B).

### Immunolocalization of *hnRNP K* in porcine testis cells

We further investigated the expression and localization of *hnRNP K* in specific cell types during testis development by immunofluorescent assay. As shown in Fig. 3A, *hnRNP K* was distributed extensively in spermatogonia and spermatocytes, and was also detected in Sertoli cells, Leydig cells and myoid cells in 3-month testis. It was noted that the numbers of spermatocytes and spermatids were increased significantly, and several layers were formed in seminiferous tubules of 4-month testis tissue. *hnRNP K* was mainly localized in the spermatocyte nucleus and spermatid membrane, showing an irregular circle, oval and triangle in Fig. 3B. The germ cell layers were decreased evidently in seminiferous tubules of 8-month testis tissues. *hnRNP K* was distributed mostly in spermatocytes and spermatids (Fig. 3C). Lastly, positive reaction signals could not be

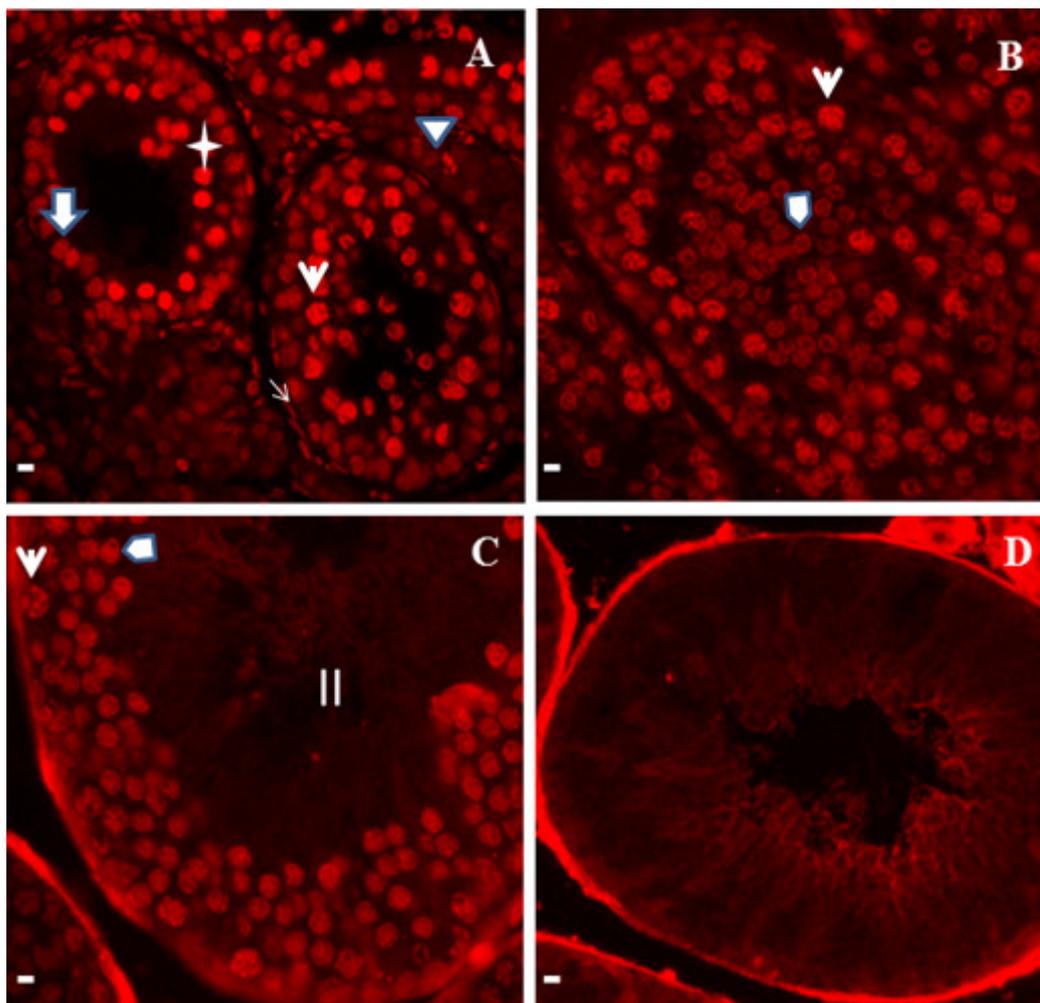


Fig. 3. Immunofluorescence localization of hnRNP K in porcine developmental testes. Panels A, B and C show sections of seminiferous tubules at 3 m, 4 m and 8 m, and Sertoli cells are indicated by thick white arrows; Spermatogonia are indicated by white asterisks; small white arrows indicate myoid cells; white triangles indicate Leydig cells; Spermatocytes are indicated by white arrow heads; Spermatids are indicated by rectangular arrows. II marks stage of seminiferous epithelium cycle. No signal was obtained when 5% BSA was used instead of the primary antibody as a negative control (Panel D). Months (m). Bars = 10  $\mu$ m.

detected in the negative control (Fig. 3D), indicating that hnRNP K was stained specifically.

#### Dynamic expression of *hnRNP K* during the development of rat testes

From the above research data, we summarized that hnRNP K was expressed highly in 3-month porcine testes, and it was mainly distributed in spermatogonia and spermatocytes. In order to understand *hnRNP K* expression levels in rat testes and to compare the expression patterns between the rat and pig, we further detected the dynamic gene expression of *hnRNP K* in the prenatal (15.5, 18.5 dpc) and postnatal (0, 7, 14, 21 and 90 dpp) testes of rat using qRT-PCR. Expression of *hnRNP K* was increased before birth (from 15.5 to 18.5 dpc). Post partum, the expression level firstly decreased with the lowest level detected at 14 dpp, and then increased with

the highest expression levels at 90 dpp (Fig. 4A). A similar expression pattern in protein level was detected (Fig. 4B).

#### Immunolocalization of hnRNP K in rat testis cells

We further examined the expression and distribution laws of hnRNP K in specific cell types during the rat testis development by immunofluorescent assay. The results showed that hnRNP K was mainly localized in gonocytes, and was also detected in Sertoli cells, Leydig cells and myoid cells in rat testis tissues of embryonic periods (Fig. 5A-B). As shown in Fig. 5C-D, hnRNP K was highly distributed in gonocytes, spermatogonia and Sertoli cells, and was also expressed in myoid cells and Leydig cells in the tubular and intertubular compartments of newborn rat testis tissues. At 14 dpp and 21 dpp, hnRNP K was mostly expressed in spermatocytes.

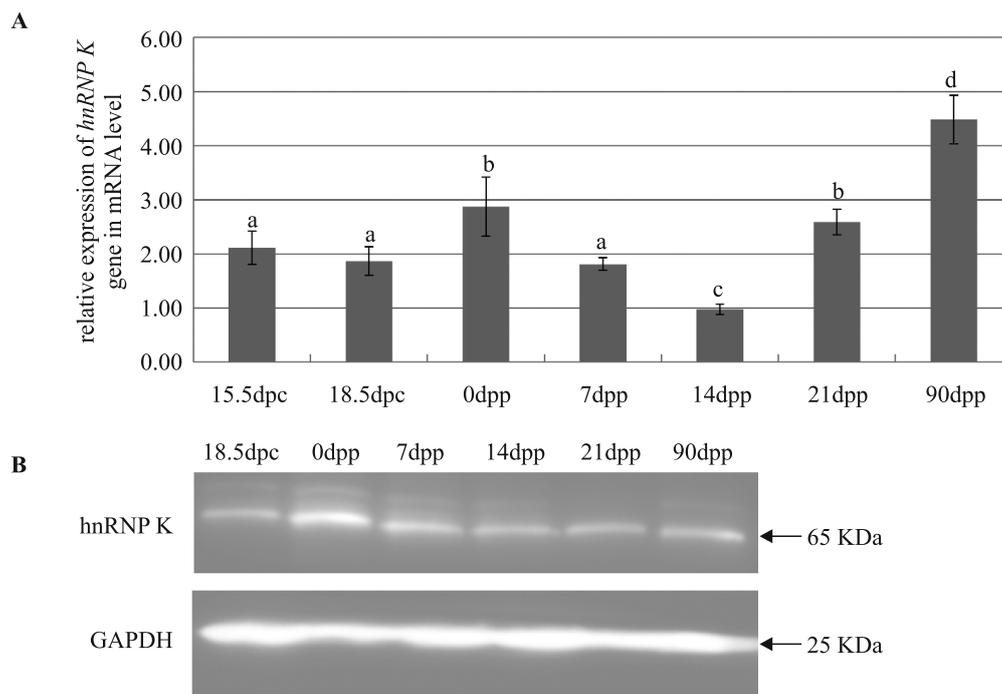


Fig. 4. Expression pattern of *hnRNP K* during rat testis development. A: mRNA expression was analyzed by qRT-PCR, normalized to *b-actin*. B: Protein expression was analyzed by western blotting, and GAPDH expression levels were used as controls. Day post coitum (dpc); day post partum (dpp).

cytes and Sertoli cells, and could be partly detected in Leydig cells and myoid cells (Fig. 5E-F). It was noted that hnRNP K was widely distributed in spermatogonia, spermatocytes, spermatids, mature spermatozoa and Sertoli cells in the VI phase of the spermatogenic cycle from 90 dpp rat testes (Fig. 5G).

## Discussion

In this study, we aimed to analyze hnRNP K expression profiles in mammal testes. We firstly analyzed the conservation of hnRNP K between the pig and rat, and the amino acids of porcine hnRNP K were 99.78 % homologous to rat (Fig. 6). These observations demonstrated that hnRNP K was highly conserved in the two species. Pigs display numerous advantages as a suitable model for research, acquire early sexual maturity (5-8 months), and have short gestational periods (114 d). However, the germ cell development and spermatogenesis in rats have been well described, and the variety in rat size and breeds provide rich materials for studies on mammalian fetal testis development (Huckins 1971, Zhao and Garbers 2002, Franca et al. 2005). The rat may therefore be another ideal model for the study of spermatogenesis.

It is well known that testes are dynamic and productive organs, which are constituted mostly by numbers of Sertoli, Leydig, peritubular myoid cells and germ cells,

and are responsible for spermatogenesis. The Sertoli cell has been shown to support and provide nutrition and a specialized environment for the developing germ cells. Peritubular myoid cells and Sertoli cells are responsible for the formation of the basal lamina and for the production of specific factors necessary for spermatogenesis (Vergouwen et al. 1991, Rebourcet et al. 2014, Rebourcet et al. 2014). Leydig cells secrete testosterone, which is very important for the maintenance of normal spermatogenesis. During testis development, the balance between Sertoli and Leydig cells, as well as the cross-talk between different germ cell types, are functionally very important for the development of the spermatogenic process (Schlatt et al. 1997, Teerds and Huhtaniemi 2015). These studies indicated that Sertoli cells proliferate actively in the fetal period, decreasing steadily after birth in rodents. However, a different pattern of Sertoli cell proliferation after birth is observed in pigs, which present two distinct and prominent mitotic phases from birth to 1 month and between 3 and 4 months. Additionally, instead of the biphasic pattern of Leydig cell development (fetal and pubertal) identified in rodents, three phases of Leydig cell development are observed in pigs (Van Straaten and Wensing 1978, Franca et al. 2000). The first occurs in the early fetal period, a striking proliferation of Leydig cells is observed during the second (perinatal) phase of Leydig cell development, and the third phase takes place from the beginning of puberty to adulthood (Mendis-

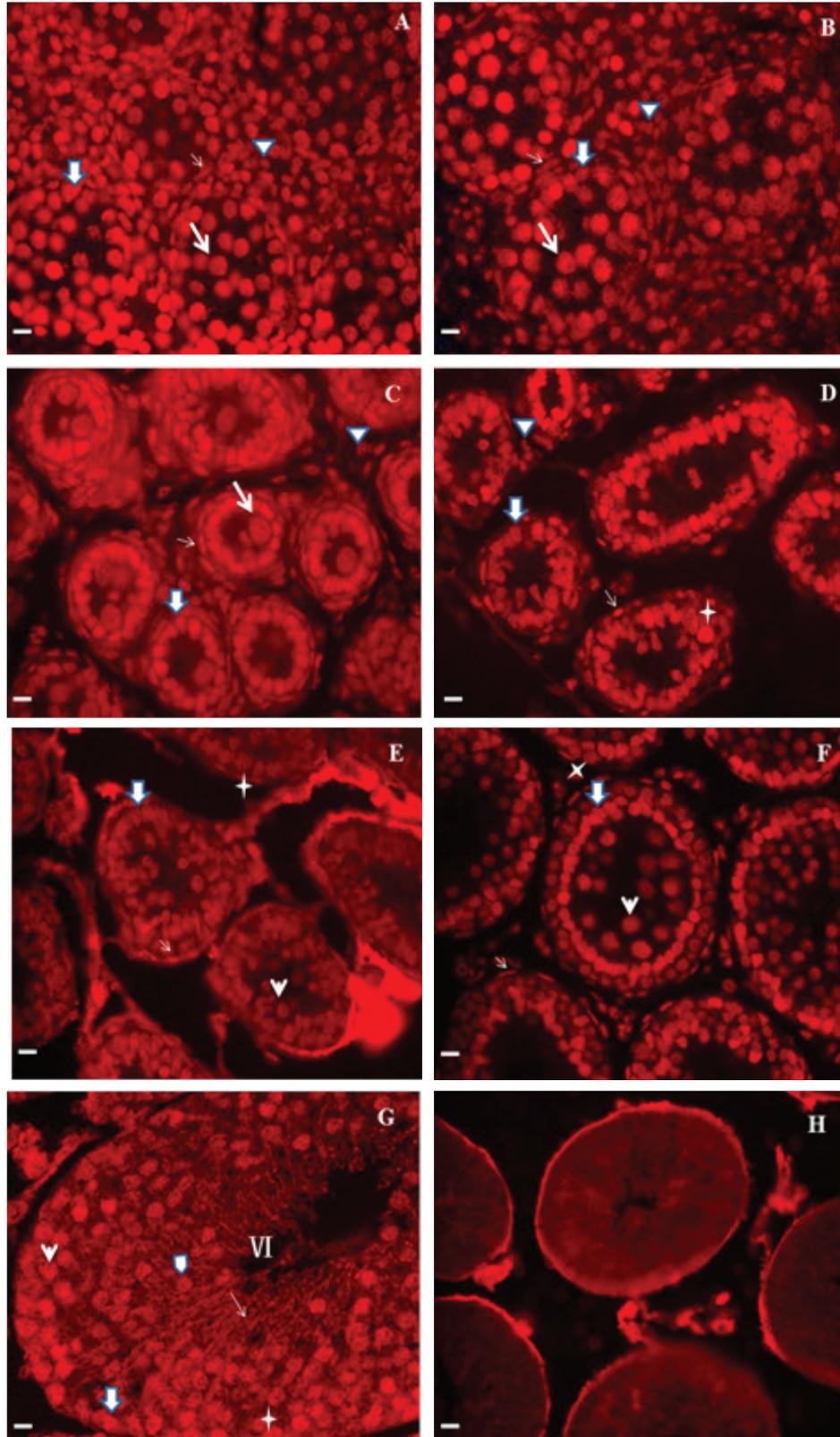


Fig. 5. Immunofluorescence localization of of hnRNP K in rat developmental testes. Panels A, B, C, D, E, F and G show sections of seminiferous tubules at 15.5 dpc, 18.5 dpc, 0 dpp, 7 dpp, 14 dpp, 21 dpp, 90 dpp of rat testis respectively. Day post coitum (dpc); day post partum (dpp). Sertoli cells are indicated by thick white arrows; Gonocytes are indicated by large white arrows; Spermatogonia are indicated by white asterisks; small white arrows indicate myoid cells; white triangles indicate Leydig cells; Spermatocytes are indicated by large white arrow heads; Spermatids are indicated by rectangular arrow; long white arrows indicate spermatozoa. VI marks stage of seminiferous epithelium cycle. No signal was obtained when 5% BSA was used instead of the primary antibody as a negative control (Panel H). Bars = 10  $\mu$ m.

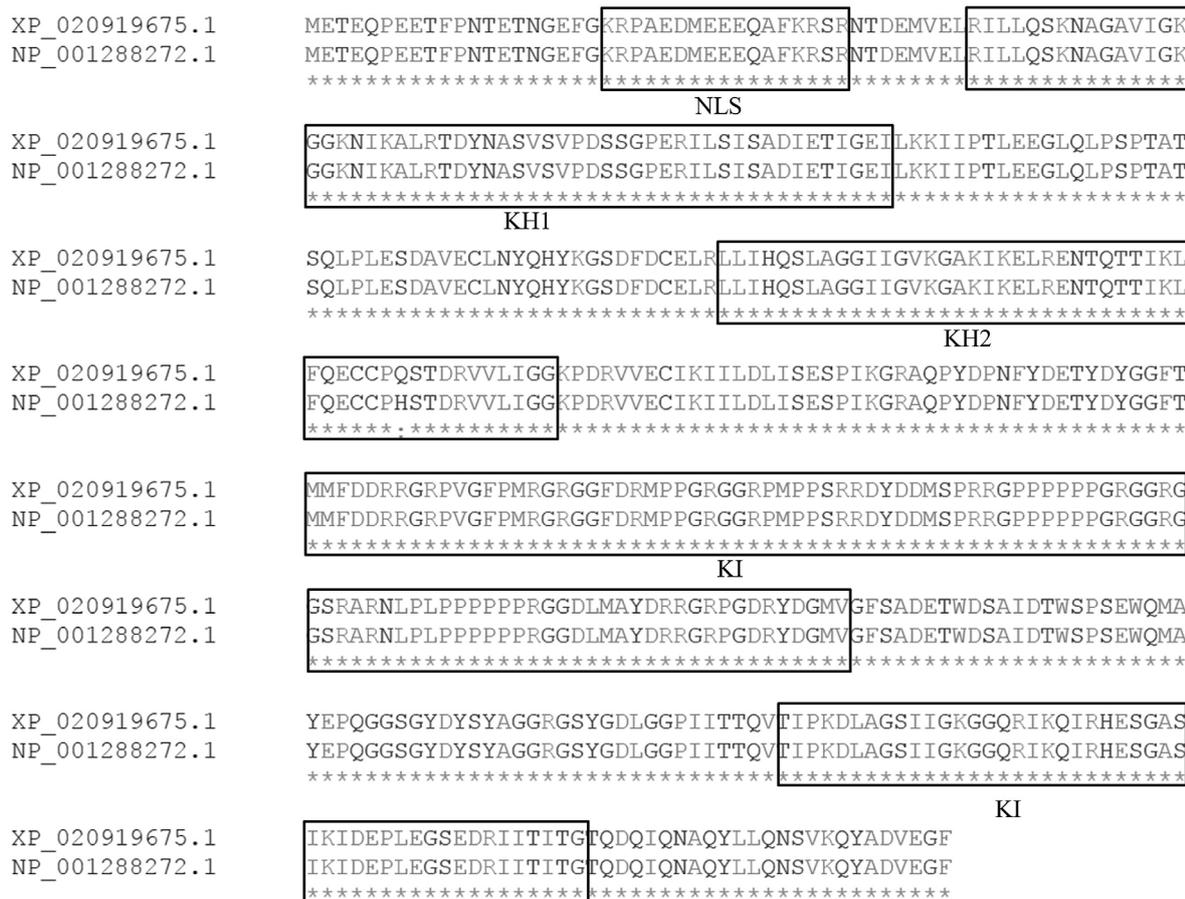


Fig. 6. hnRNP K protein sequence comparison between pig and rat.

-Handagama and Ariyaratne 2001, Lervik et al. 2013). HnRNP K is a major cause of protein diversity and has critical roles in cell proliferation by promoting c-myc transcription and inhibiting p21 translation (Lu and Gao 2016). The higher hnRNP K expression levels are coincident with the periods of more prominent Sertoli cell and Leydig cell proliferation. As detected in the testes, hnRNP K expressed generally high in pigs and rats, the expression patterns of hnRNP K in the early developing testes were relatively ubiquitous, and were also robust. This most likely reflects the requirement of hnRNP K in testis development and initiation of spermatogenesis in mammalian species.

Further comparison of hnRNP K expression patterns in tissues and cells between pigs and rats were conducted. Within the limited development stages in the two mammalian models, we point out three important observations: firstly, hnRNP K expressed highly in the early developing testes; secondly, hnRNP K localized in germ cells and somatic cells with a broad pattern; thirdly, hnRNP K distributed differently with germ cell types, evidently in the nucleus of gonocytes, spermatogonia, spermatocytes and in the membrane of spermatids. These patterns may be explained firstly by

the conservatism of species from pigs and rats; additionally, hnRNP K is a multifunctional protein participating in transcription, translation, RNA splicing, DNA repair and chromatin remodeling biological functions, which will be crucial for the survival of the organism (Barboro et al. 2014, Gallardo et al. 2016). Previous research also indicated that hnRNP K was highly expressed in spermatogonia and pachytene spermatocytes during mouse spermatogenesis (Gan et al. 2013). Moreover, hnRNP K was expressed in the cytoplasm of spermatogonia, as well as in the nuclei of spermatocytes and round spermatids (Yu et al. 2011). These studies, combined with our results, suggest that hnRNP K may have an important role in spermatogenesis.

In this study, hnRNP K was expressed in a defined, spatiotemporal manner during the development of testes. Our data support the idea that the multifunctional RNA-binding protein hnRNP K was expressed in a specific pattern at different developmental stages of the testes during spermatogenesis, especially localized in different cell compartments, which implied specific and potentially nonredundant roles for this gene in mammalian spermatogenesis.

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