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Molecules and Cells

Ankrd7, a Novel Gene Specifically Expressed in Sertoli Cells and Its Potential Roles in Sertoli Cell Maturation

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The somatic Sertoli cells play an essential role in testis determination and spermatogenesis by providing nutrition and structural support. In the current study, we report on the novel Ankrd7 gene that contains five ankyrin repeat domains. This gene was specifically expressed in Sertoli cells and was regulated in a maturation-dependent manner. Its expression was restricted to testicular tissue, and its mRNA could be detected in testes at as early as 14 dpp (days post partum) using RT-PCR analysis. In both testicular tissue sections and *in vitro* cultured Sertoli cells, the Ankrd7 protein was localized to the nucleus of the Sertoli cell. Immunohistochemistry and immunocytochemistry investigations showed that the protein was detectable in testicular tissues at 20 dpp, at which time Sertoli cells were gradually differentiating into their mature cellular form. These results suggest that Ankrd7 is probably involved in the process of Sertoli cell maturation and in spermatogenesis.

INTRODUCTION

Spermatogenesis in mammals is a complex process that is dependent upon the optimal functioning of a specific testicular cell type, the Sertoli cells. These cells serve as nurse cells and play an essential role in the process of sexual differentiation and spermatogenesis. A tumor-suppressor gene, WT1 (Wilms' tumor 1 transcription factor), is expressed in Sertoli cells from the embryonic stage to adulthood and is required for testis development and spermatogenesis. Knockout studies using wt1 knockout or conditional knockdown mice have shown that suppression of this factor results in disruption of sexual differentiation, depletion of germ cells and male infertility (Gao et al., 2006; Rao et al., 2006). The number of Sertoli cells in the adult testis determines the testis size and germ cell number because the number of germ cells that each Sertoli cell is able to support is fixed (Orth et al., 1988).

Previous studies have also indicated that the stem cell factor (SCF) produced exclusively by Sertoli cells is also crucial for spermatogenesis. This factor apparently acts by binding to the c-kit receptor expressed on type A spermatogonia (Mauduit et al., 1999). The SCF/c-kit system is believed to regulate germ cell proliferation through a combination of an apamycin-sensitive PI3-K/AKT/p70S6K/cyclin D3 pathway, meiosis, and apoptosis (Feng et al., 2000). The functions of FSH, a key endocrine hormone with major actions on reproduction, are also regulated via Sertoli cells. Only Sertoli cells in testicular tissue express the FSH receptor; germ cells have neither androgen nor FSH receptors (Walker and Cheng, 2005).

From the neonatal period, through puberty, and into adulthood, the Sertoli cells gradually mature and differentiate in both morphology and in their functional aspects. After birth, Sertoli cells continue to proliferate and differentiate until the beginning of puberty, when they cease dividing and start instead to nurse the germ cells. Prior to puberty, the Sertoli cells are immature and differ considerably with respect to morphology and biochemical activity from the mature cells (Petersen and Soder, 2006). Around the onset of puberty (15 to 30 days after birth in mice), Sertoli cells undergo a radical change from an immature, proliferative state to a mature, non-proliferative state. Adjacent Sertoli cells form tight junctions (TJs), which creates the blood-testis barrier (BTB) and provides a specialized and protected environment within the testis. This includes a unique adluminal compartment in which the meiotic and post-meiotic steps of spermatogenesis can proceed, as well as allowing formation of a fluid-filled lumen for germ cell development (Cheng and Mruk, 2002).

Hormones, in particular FSH, as well as thyroid hormones, growth factors and paracrine regulators are involved in the switching of Sertoli cells from the immature to the mature stage. Protein markers of maturity and immaturity include anti-Mullerian hormone, aromatase, cytokeratin-18, GATA-1, laminin alpha5, M2A antigen, p27kip1, sulphated glycoprotein 2, androgen receptor and Wilms' tumor gene (Sharpe et al., 2003). However, despite the knowledge of numerous protein changes occurring in this cell during its differentiation to the mature stage, the actual mechanism regulating the maturation of the Sertoli cell has remained obscure.

Proteins containing ankyrin repeat domains, tandem repeats of about 33 amino acids, constitute a large number of functionally diverse proteins found mainly in eukaryotes (Bork, 1993).

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Defects or deficiency of ankyrin on the short arm of chromosome 8 (8p11) are thought to be responsible for hereditary spherocytosis (HS) (Lux et al., 1990). In this paper, we report on the expression and localization of Ankrd7, a novel Sertoli cell-specific protein containing ankyrin repeat domains, and investigate its potential role in Sertoli cell maturation.

MATERIALS AND METHODS

Experimental animals and reagents

Adult CD-1 mice were obtained from the Experimental Animal Center, Chinese Academy of Sciences. All animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals and all protocols were approved by the Committee of Animal Care and Use of the Institute of Zoology, Chinese Academy of Sciences. Reagents for the cell cultures and the animal treatment were all obtained from Invitrogen (USA), Beijing Chemical Reagents Incorporation (China) and Sigma (Sigma-Aldrich, USA) unless specified otherwise.

RNA isolation and RT-PCR

Total RNAs from various mouse tissues and testes at different stages of development were extracted for RT-PCR analysis. The primer pairs 5′-AAGAAGTTCTTCCCCTTCAG-3′ and 5′-AGTAAGCTGTTTGCTCTG-3′ were used to detect Ankrd7 mRNA expression. Briefly, total RNAs (5 μg) were used as templates for reverse transcription using Superscript III (Invitrogen). Detailed procedures were performed according to the manufacturer's instructions. For PCR, the reaction mixture was first heated at 94°C for 2 min, followed by 30 cycles of denaturing at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 45 s. The reaction was terminated by a final extension step at 72°C for 5 min.

Recombinant protein expression and polyclonal antibody production

The mouse Ankrd7 cDNA fragment encoding the N-terminal 116 amino acids (1-116) was subcloned into the pGEX-4T1 vector (Pharmacia Biosciences). The GST fusion protein was then expressed in *Escherichia coli* strain BL21 and purified using a GSTrap FF column (Amersham Pharmacia) according to the manufacturer's instructions. Next, purified protein of 500 μg was emulsified in complete Freund's adjuvant and injected into healthy rabbits. Three boosting injections with 500 μg protein emulsified in incomplete Freund's adjuvant were then given at 3-week intervals. Antibody was purified from serum via affinity purification on a protein G column.

Preparation and culture of Sertoli cells

Mouse Sertoli cells isolated at different stages of development were prepared by a two-step enzymatic digestion and cultured as earlier described, with slight modifications (Karl and Griswold, 1990; Shi et al., 2006). Briefly, decapsulated testis tissue was treated with 10 volumes of Digestion solution I (2 mg/ml collagenase type IV and 200 mg/ml DNAse (Sigma-Aldrich) in D-PBS) at room temperature for 3 to 5 min with gentle agitation, followed by 3 washes with 10 volumes of PBS. Collected specimens were then treated with 5 volumes of Digestion solution II (2 mg/ml collagenase type IV, 200 mg/ml DNAse and 2 mg/ml hyaluronidase (Calbiochem) in serum-free DMEM) at room temperature for 2 to 5 min with vigorous agitation until the tubular clumps were invisible. After washing 3 times in PBS, the dissociated cell suspension was filtered through a 60 μm nylon mesh. Cells were washed twice by centrifugation at 200 × *g* for 5 min and were suspended in DMEM medium containing 10% FBS. The pellet was finally resuspended in DMEM/F12 culture medium supplemented with 10% FBS, 2 mM L-glutamine, 100 unit/ml penicillin and 100 mg/ml streptomycin and plated on 0.2% (w/v) gelatin-coated tissue culture flasks at a density of 2 × 10^5 cells/cm^2. Cells were cultured at 37°C in an atmosphere of 5% carbon dioxide in air for 1 h. After gentle agitation, floating cells were removed by changing the medium, and the cells that remained attached to the bottom were again incubated overnight. The next morning, the cells were detached with trypsin-EDTA solution and passaged into a fresh dish to remove germ cell contamination.

Immunohistochemistry and immunocytochemistry

Immunohistochemistry was performed as described previously, with some modifications (Alsheimer et al., 2000). Briefly, mouse testicular tissues were snap-frozen and 8 μm sections were fixed immediately in 4% paraformaldehyde for 15 min at room temperature. After blocking, the sections were incubated with affinity-purified Ankrd7 antibody (diluted at 1:400 in blocking buffer). Pre-immune rabbit serum, used as a negative control, was incubated with control sections for 1 h at room temperature. Secondary antibody was FITC-conjugated anti-rabbit (1:500) from Jackson Laboratories (Cambridgeshire, UK). The nuclei were stained by DAPI at a final concentration of 1 μg /ml for 10 min at room temperature. When mouse testis sections were performed to double-staining with anti-Ankrd7 and anti-Wt1 antibodies, TRITC-conjugated anti-mouse (1:500) antibody was used as a secondary antibody for anti-Wt1 antibody.

Sertoli cells at 90% confluence cultured on glass cover slips were then fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.2% Triton X-100 for 10 min at room temperature. The procedure for staining with anti-Ankrd37 or anti-Wt1 antibody was performed as the immunohistochemistry assay demonstrated. After washing with PBS, a 10 min staining of the nuclei was performed using DAPI at a final concentration of 1 μg/ml. The images were captured using a fluorescence microscope (Nikon).

RESULTS

Structural features of Ankrd7

In the search for testis-specific genes, the Ankrd7 gene was identified in the NCBI Unigene database (UniGene entry: Mm.67665, GenBank entry: NM_029202), as its ESTs are only distributed in the testis. Ankrd7, which contains five ankyrin repeat domains, has a full length of 840 nucleotide bases encoding a putative protein of 279 amino acids, and shows no putative subcellular localization signal. A schematic representation of the putative structural features of Ankrd7 is shown in Fig. 1A. BLAST searches indicated sequence homology in human (*Homo sapiens*, NP_062618), rat (*Rattus norvegicus*, XP_578232), chimpanzee (*Pan troglodytes*, XP_519333) and chicken (*Gallus gallus*, XP_001233934) Ankrd7 genes. The identity of Ankrd7 between mouse and human, rat, chimpanzee and chicken was 50%, 89%, 49% and 35%, respectively, as determined by the EBI clustalw web tool. Sequence identity between mouse and rat was much greater than that between mouse and other species, suggesting that evolutionary divergences existed (Fig. 1B).

The expression of Ankrd7 was restricted to mouse testes

To investigate Ankrd7 expression pattern in various tissues, multiple tissue RT-PCR and Western blotting were performed. Total RNA and cDNA were generated from mouse testis, epididymis, ovary, uterus, liver, stomach, lung, muscle, skin, spleen,

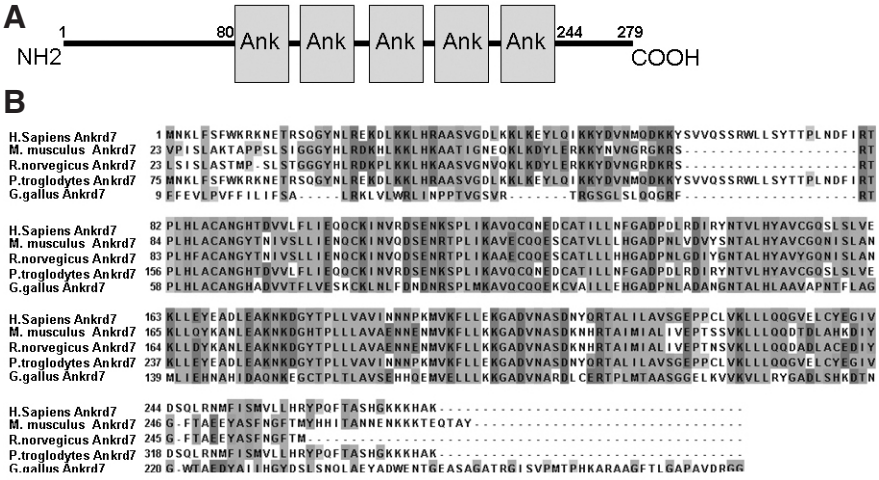

Fig. 1. (A) A schematic representation of putative structural features of Ankrd7 proteins. Ankrd7 is a putative protein consisting of 279 amino acids and containing five ankyrin repeat domains but no subcellular localization signal. Its presumed molecular mass is 31 kDa. (B) The amino acid sequence alignment of Ankrd7 homologies. Sequence alignment of human (*H. sapiens*, NP_062618), mouse (*M musculus*, NP_083478), rat (*R. norvegicus*, XP_578232), chimpanzee (*P. troglodytes*, XP_519333) and chicken (*G. gallus*, XP_001233934) was performed using the EBI clustalw web tool.

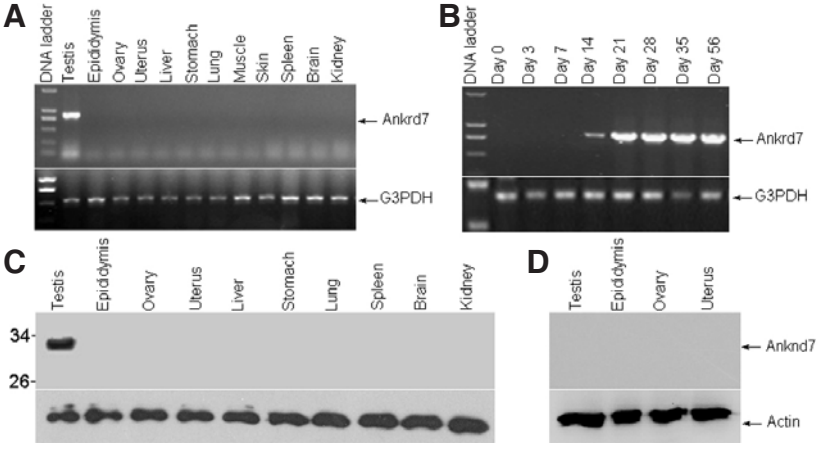

Fig. 2. (A) The expression of the Ankrd7 gene was restricted to mouse testicular tissue as shown by the RT-PCR assay. (B) Ankrd7 mRNA was first detected in testis at 14 dpp (days post partum). (C) Multiple mouse tissues were assayed by immunoblotting with affinity-purified rabbit anti-Ankrd7 antibody, which showed that this protein was restricted to the testis. (D) Specificity of Ankrd7 antibody. Incubation with Ankrd7 immunizing peptide overnight at 4°C showed no positive staining in immunoblotting assays. Expression of G3PDH and beta-actin served as controls.

brain, and kidney. Ankrd7 was expressed in a testis-specific manner (Fig. 2A). Its mRNA expression was first detected in the mouse testis at 14 dpp, and its expression was sustained thereafter in the adult testis (Fig. 2B). The testis-specific expression pattern was confirmed by multiple tissues Western Blot detection using the polyclonal rabbit antibody to Ankrd7 purified in this study (Fig. 2C). The specificity of this Ankrd7 antibody was tested by incubation with Ankrd7 immunizing peptide overnight at 4°C, which revealed no positive staining in immunoblot assays (Fig. 2D). As shown by the data in the upper figure, Ankrd7 was abundantly expressed in the testis in a tissue-specific and a developmentally regulated manner.

Ankrd7 protein was localized in Sertoli cells in developing mouse testis

Immunohistochemical assays indicated no positive immunofluorescence staining for Ankrd7 in mouse testis at 10 dpp (Fig. 3A). However, at 20 dpp (Fig. 3B), specific staining was observed in the nuclei of the Sertoli cells. This staining signal intensified until adulthood and then remained at a sustained level in the adult testis (Figs. 3C and 3D). Wt1, a specific and stable marker of Sertoli cells, has been localized to the nuclei and its expression remains at a constant level during the process of mouse testis development (Mackay, 2000). Therefore, we used wt1 as a marker to detect and verify the localization of Ankrd7 protein in the Sertoli cell. When frozen testis sections were double-stained with anti-Ankrd7 and anti-wt1 antibodies, both Ankrd7 and wt1 were found to be co-localized in the nuclei of the Sertoli cells (Fig. 4).

Ankrd7 protein was expressed in a maturation-dependent pattern in Sertoli cells

Previous investigations have shown that mouse Sertoli cells gradually differentiate into mature cells at puberty (15-30 days after birth in the mouse) (Sharpe et al., 2003). The result of immunocytochemistry assays in the current study showed that no expression of Ankrd7 was detected in Sertoli cells cultured *in vitro* until they had reached 20 days of age (Fig. 5). This finding was coincident with the results observed in frozen testis sections as above. Protein expression of Ankrd7 and wt1 again showed a precise co-localization in the nuclei of the *in vitro* cultured Sertoli cells (Fig. 5).

DISCUSSION

Spermatogenesis, the generation of haploid spermatozoa in testicular seminiferous tubules, is a complicated process that involves cellular mitotic and meiotic divisions, morphological changes, and apoptosis. The somatic Sertoli cells play an essential role in this process. In the current study, we report on a novel gene, Ankrd7, that contained five ankyrin repeat domains and that was specifically expressed in the nuclei of Sertoli cells.

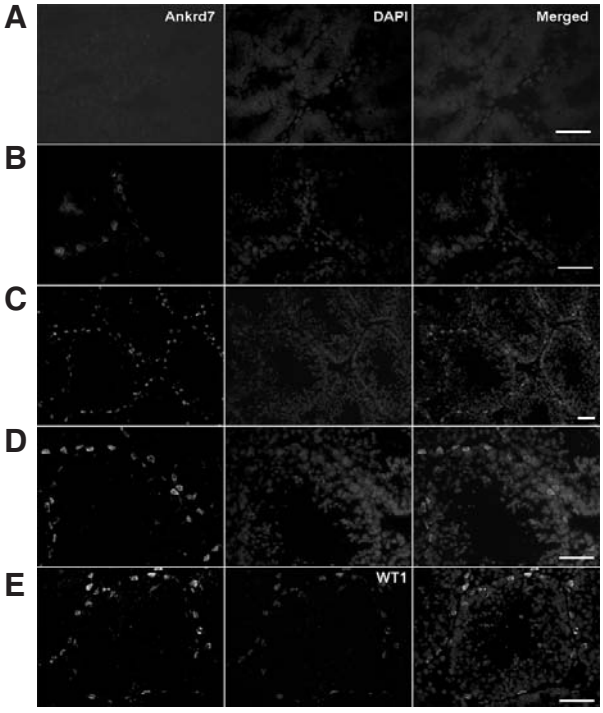

Fig. 3. (A) Frozen testis sections stained with affinity-purified anti-Ankrd7 antibody. No positive and specific immunofluorescence staining was seen in mouse testis at 10 dpp (days post partum). (B) At 20 dpp, the specific staining was first seen in the nuclei of the Sertoli cells. (C, D) The expression of Ankrd7 was sustained in the adult testis. (E) Frozen testis sections were double-stained with anti-Ankrd7 and anti-wt1 antibodies. The same sections were counterstained for DNA with DAPI, and the signal was merged with the antibody signals. Bars represent 40 μm.

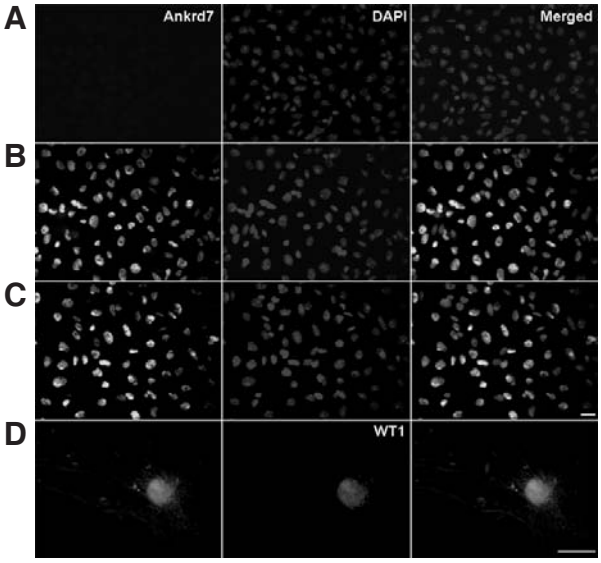

Fig. 4. (A) No positive and specific immunofluorescence staining was detected in Sertoli cells at 10 dpp cultured *in vitro*. (B) Ankrd7 expression was first detected in the nuclei of Sertoli cells at 20 dpp cultured *in vitro*. (C) The expression was sustained in the nuclei of mature Sertoli cells cultured *in vitro*. (D) Sertoli cells cultured *in vitro* at the mature adult stage and double-stained with anti-Ankrd7 and anti-wt1 antibodies. The two proteins co-localized to the nuclei of Sertoli cells. The cells were counterstained for DNA with DAPI, and the signal was merged with the antibody signals. Bars represent 20 μm.

The ankyrin repeat domain exists in a large number of functionally diverse proteins, primarily from eukaryotes, and is one of the most common motifs mediating protein-protein interactions in nature. Many proteins possessing ankyrin repeat domains are reported to be involved in a number of diverse biological functions such as signal transduction, transcriptional regulation, differentiation and apoptosis (Debrincat et al., 2007; Ferguson et al., 2007; Li et al., 2007; McDaneld and Spurlock, 2008).

Searching for an interacting target using the yeast-two hybrid system has often been shown to provide valuable information about the function of novel genes. We screened the mouse testicular Ankrd7 for potential interacting proteins using a yeast-two hybrid assay, but unfortunately no protein was identified in our experiment (data not shown).

As puberty approaches, the somatic Sertoli cells undergo a gradual change from an immature, proliferative state to a mature, non-proliferative state with characteristics of morphological and functional switches. Morphologically, the Sertoli cells elongate into the adjacent cells and tight junctions are established. These tight junctions create a unique adluminal compartment in the seminiferous tubules in which spermatozoa are incessantly produced via germ cell meiotic and post-meiotic processes (Sharpe et al., 2003). Functionally, the mature Sertoli cells alter their pattern of protein expression and begin to produce transferrin (Skinner and Griswold, 1980) and the inflammatory cytokine IL-1 (Sultana et al., 2000; Wahab-Wahlgren et al., 2000). At the same time, the production of cytokeratin, anti-Müllerian hormone, aromatase, and inhibin beta subunit is halted (Andersson et al., 1998; Petersen and Soder, 2006). The current study showed that Ankrd37 was localized to the Sertoli cell nuclei both in testicular tissue sections and in cultured Sertoli cells *in vitro*. No expression of Ankrd7 was detected in Sertoli cells from either *in vitro* cultured or testicular tissue until 20 days of age, at which time the Sertoli cells had differentiated into their mature form. Ankrd7 is therefore apparently spatially and temporally regulated in the mouse testis during development, which suggests that Ankrd7 might be involved in the process of Sertoli cell maturation.

Various endocrine and paracrine factors are concerned with the process of Sertoli cell maturation. FSH is the major endocrine hormone known to regulate Sertoli cell proliferation and maturation. FSH, testosterone and the testosterone receptor are absolutely indispensable for the formation of the tight junctions and for an adequate blood-testis barrier function. AR has been reported to first appear in Sertoli cells just before the final maturation of the cell and has been suggested for use as a marker of mature Sertoli cells (Meng et al., 2005). The tight junction formation and functional integrity of blood-testis barrier are one of the most important characteristics of Sertoli cell maturation. In addition, many paracrine growth factors, including insulin-like growth factors I and II (IGF- I and IGF- II) (Borland et al., 1984), fibroblast growth factor (FGF) (Cancilla and Risbridger, 1998) and transforming growth factor-α (TGF-α) (Petersen et al., 2001) also play important roles in regulation of Sertoli cell proliferation and maturation.

In conclusion, Ankrd7 was specifically expressed in the nuclei of Sertoli cells in a maturation-dependent manner. However, no interacting proteins could be identified in the current study.

The results suggested that Ankrd7 plays an important, but as yet precisely undetermined, role in Sertoli cell maturation and spermatogenesis. Further investigation is required to elucidate its full role in spermatogenesis, and the data presented here provide some clues for the direction of future efforts into elucidating the functions of Ankrd7.

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