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Original Article

Androgen receptor expressed in three testicular cells and *Ar*-spermatogenesis was arrest at the zygotene stage

Kai Deng[1,3], Xin Chen[1,3], Changjun Zhang[1,3], Xiaoyan Wang[1,2,3]

[1]Reproductive Medicine Center, Renmin Hospital, Hubei University of Medicine, Shiyan, China; [2]Department of clinical Oncology, Taihe Hospital, Hubei University of Medicine, Shiyan, China; [3]Biomedical Research Institute, Hubei University of Medicine, Hubei, China

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Abstract: Objective: To investigate the expression and distribution of Androgen Receptor (AR) in all kinds of testicular tissue cells, as well as its effect to reproductive system. Methods: Using immunofluorescence to detect AR expression and distribution in mice testis cells. By constructing *Ar* knockout mice and observing its reproductive system phenotype to identify how AR affect the reproductive system. Using chromosome spreads to detect which stage the spermatogenesis was arrest in *Ar* knockout mice. Results: AR have a strong signal in Sertoli cells, Peritubul myoid cells and Leydig cells but without any specific signal in Germ cells. *Ar* knockout mice changed penis position compared to control and the distance of its perineum became shorter, just seems like fake clitoris. The testicular volume of *Ar* knockout mice was significantly smaller and the cell species, distribution and proportion within the seminiferous tubules changed significantly. *Ar*-null mice had large numbers (over 70%) of spermatogenic spermatocytes compared to the wild type mice and had more than 20 centromeres. Conclusion: *Ar* expressed in testis Sertoli cells, Peritubul myoid cells and Leydig cells except in germ cells. *Ar* knockout mice appeared feminization of male and its sperm blocked during the early phase of the first meiosis. Spermatogenesis was arrest at the zygotene stage in *Ar* knockout mice because the homolog pairing was disturbed in *Ar*-null mice.

Keywords: Androgen receptor, *Ar* knockout, feminization of male, spermatogenesis, zygotene stage

Introduction

As an important event in the process of spermatogenesis, meiosis could make sure homologous chromosomes to complete chromosome recombination and to produce the haploid male gametes. The abnormality of meiosis may lead to infertility and aneuploidy. Previous research shows that infertility affects about 15% of couples at childbearing age, and male infertility accounted for more than 50%, of which 7% of patients are due to spermatogenesis abnormalities.

Spermatogenesis is a highly complicated and tightly controlled process of germ cell proliferation and differentiation. Spermatogenesis can be roughly divided into three stages from sperm stem cells proliferation eventually to become the mature sperm [1]. The first stage is the sperm stem cells mitosis into spermatocyte, and then the spermatocytes become haploid round sperm after meiosis. The final stage is sperm cells further deformation, and circular specialized into mature sperm [2]. Spermatogenesis is highly dependent on autocrine and paracrine regulation of all kinds of testicular tissue cells, it is mainly composed of the sertoli cells (SCs), peritubular myoid cells (PM), leydig cells (LCs) and germ cells (GCs) [3]. The first wave of sperm production cycle is about four to five weeks after the mouse was born, and its three phases respectively need 11, 10, 14 days or so [4].

Androgen plays an important role in spermatogenesis regulation, and testosterone is one of the most important steroid hormones in androgen that secreted by the interstitial cells. SCs is the mainly target cells of testosterone signals

[5]. Testosterone plays an important role in regulating meiosis and other stage of spermatogenesis [6]. As a intermediate for adjust the androgen effects, Androgen Receptor mainly exist in the SCs, PM and LCs, but there is a controversy whether it also exists in GCs [7]. Our experiment will determine the distribution of AR in testicular tissue cells by immunofluorescence, and then discuss its function in the reproductive system.

Mouse nuclear genetic material has 20 pairs of chromosomes. The spermatocytes divide only one time during the division, but the cells divide two times consecutively (meiosis I and meiosis II). The homologous chromosomes separate during meiosis I and then form the secondary spermatocytes. The sister chromatids separate during meiosis II and form the haploid round sperms. The two meiosis split the homologous chromosome with the sister chromatids to the daughter cells, so that the gamete chromosomes contain a half chromosome of the mother cell to ensure a stable genetic inheritance.

According to the different chromosome morphology, prophase I can be divided into five stages: Leptotene stage, zygotene stage, pachytene stage, diplotene stage and diakinesis stage. The chromosomes are quite long and thin in leptotene stage and each chromosome is composed of two sister chromatids. The homolog pairing and synapsis will occur between homologous chromosomes in zygotene stage. The non-sister chromatid of homologous chromosome form chiasmata in pachytene stage and complete the gene recombination. However, each chromatid still has the same gene. The chiasmata will separate in diplotene stage. The homologous chromosomes were further condensed and separated in diakinesis stage. The recombination point disappeared and finally the spindle formed. In this process, each fine subcellular structure and important cell events can lead to defects in sperm abnormalities and eventually lead to male infertility.

Ar gene located on the X chromosome q11-12 districts, composed by eight exons and seven introns, and encoded 919 amino acids [8]. The *Ar* gene knockout mice model of the compared system is based on the zinc finger nuclease's (ZFN-Zinc Finger Nuclease) gene knockout technology which can make the fixed point breakdown on the target genes and significantly improve the efficiency of homologous recombination [9]. It is a new type of high efficient gene targeting technology [10]. In this experiment, we will use ZFN technology to knockout *Ar* gene in mice and then investigate the effects on the reproductive system by observing the changes of *Ar* gene knockout mice phenotype.

Material and methods

Experimental animal

The *Ar*-null male mice birth from *Ar*-null female mice mated with adult C57BL/6J male mice.

The establishment of the Ar knockout mice and its efficiency detection

Ar gene knockout female mice was made by zinc-finger nucleases (ZFN) mediated gene targeting recombinant technology. Due to missing four base pairs of the first exon, the coding sequence exhibits frame shift mutation. Therefore, it can neither produce normal mRNA, nor translate AR protein.

Authenticating method: The DNA extracted from cracking rat-tail can used for genotype identification. Due to the destroyed AvaI restriction enzyme of knocked-down mice, there exists only a complete fragment of PCR products. However, in the wild type, two short clips will appear after enzyme digestion.

Tissue paraffin embedding

The mice testicle collected and placed in 4% paraformaldehyde for fixation and subjected to standard processing. The samples were incubated overnight at 4°C, then dehydrated in graded alcohol, soaked it with 70% alcohol for 1 hour, then 80% for 1 hour, next 95%. Transferred the tissue into the absolute ethyl alcohol 30 minutes for twice, then wash the tissue with 100% ethanol and xylene mixture (1:1), soaking for 30 minutes, changed into xylene for another 30 minutes. Later, wax dip the tissue in the 1:1 mixture of xylene and paraffin for 1 hour. Put the biological tissue into paraffin wax 1 and 2 for soak 1 hour of each. The next step is embedding and sectioning, the thickness of the slice is 5 microns/piece. Finally, baking the tissue slice overnight at a temperature of 37°C.

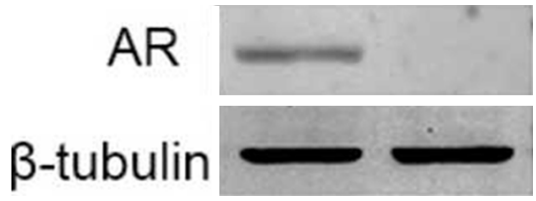

Figure 1. Effect appraisal about *Ar* knockout. There was no androgen receptor expression in *Ar*-null mice.

Immunofluorescence colony staining

Dewaxing the tissue and make it rehydration, soaking the paraffin section by dimethyl benzene xylene I, II, III for 10 min, respectively. Then rehydration with the gradient alcohol (100%, 100%, 95%, 80%, 70%) 5 min of each, wash the rehydration samples in 1 × PBS for 5 minutes, then repeat this step for 3 times. Antigen retrieval was conducted by boiling the slide in sodium citrate (4.5 ml 0.1 M citrate, 20.5 ml 0.1 M sodium citrate, volume to 250 ml with deionized water) for 4~5 minutes, restore for 15 minutes, and natural cooling to room temperature. Washing with 1 × PBS three times, 5 minutes each time, then blocking with antigen, 5% donkey serum, which has diluted with PBST, and blocking at room temperature (28°C) for 45~60 minutes in humidified box; add anti-androgen receptor antibodies that has been diluted with 0.3% Triton X-100 for incubation in the humidified box overnight at 4°C in darkness, wash with 1 × PBS three times, 10 minutes each time; then incubate with secondary antibody FITC/TRITC (1:200) + DAPI (1 mg/ml, 1:200) in the humidified box at room temperature for 1 hour, wash with 1 × PBS three times, 10 minutes each time. Add resistance to fluorescence quenching agent and observe after sealing piece by the fluorescent microscope.

Protein immunoblotting (Western blot)

For detecting the proteins, mice sacrificed and take the tissue in a centrifuge tube, then dissociated the cells for 30 minutes in low temperature with moderate cell lysis buffer RIPA. Centrifuging and collecting the supernatant and determination the concentration of protein. The proteins were separated by SDS-PAGE, 40 μg for each well. Put the gel and nitrocellulose (NC) membrane into the transfer buffer for 10 minutes and transferred for 2.5-3 hours. Incubating the NC membrane at room temperature with blocking solution (5% skim milk powder ,0.1% Tween-20) for 1 hour. Then incubating the membrane with anti-androgen receptor antibody and goat anti-mouse antibody at room temperature for 1 hour in turn and wash with 0.1% PBST three times, 10 minutes each time.

Chromosome immunofluorescence (chromosome spreads)

Take the mice testis and put it in 1 × PBS, remove the tunica albuginea and transfer into hypotonic solution for 30~60 minutes until the seminiferous tubule scatter. Take seminiferous tubule that 3 cm length in 20 μL 100 mM sucrose solution, use tweezers to break them into small flake, and then add sucrose solution to 40 μL. Blowing it before drop on the slide that treated with 1% paraformaldehyde and drying on room temperature. Then blocking with antigen, 5% donkey serum, which has diluted with PBST, and blocking at 28°C for 45~60 minutes in humidified box; incubating with primary antibodies that has been diluted with 0.3% Triton X-100 at 4°C overnight, wash with 1 × PBS three times, 10 minutes each time; then incubate with secondary antibody FITC/TRITC (1:200) + DAPI (1 mg/ml, 1:200) for 1 hour, wash with 1 × PBS three times, 10 minutes each time. Add resistance to fluorescence quenching agent and observe after sealing piece by the fluorescent microscope.

In vitro cultivation of testicular tissue

Preparing 1.5% agarose with 5 mm thickness, then cutting into pieces of 10 × 10 mm. putting in the six holes culture plate with 3 pieces agarose for each hole. Then add the nutrient solution: the basic culture medium DMEM/F12, FBS 10%, HGF 5 ng/ml, Activin-A 100 ng/ml, hFSH 200 ng/ml, testosterone 1 μM, hBMP 420 ng/ml, hBMP 720 ng/ml. Execute the mice with cervical dislocation method, and take the testis in the petri dish containing 1 × PBS. Cut the tissue into pieces about 1-3 mm. Tile the tissue on the agar and cultivate in the 33°C, 5% CO_2 incubator, with the Gas-liquid plane method. The next day replace the culture solution.

Statistical analysis

Each set of data are from three independent experiments, and was reported as the mean ± SE with computer analyzed using SPSS 19.0

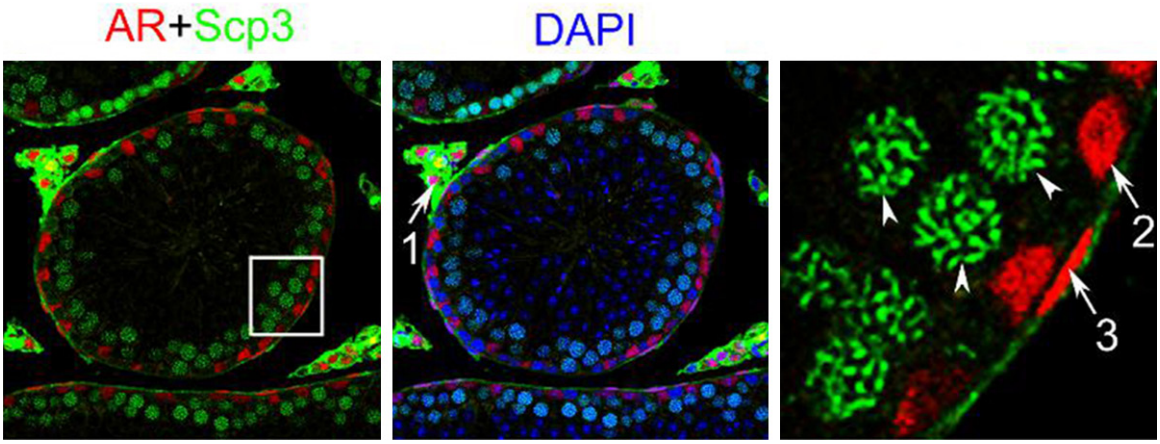

Figure 2. Immunofluorescence analysis of adult mice testicular tissue. The AR protein(red) was specific dyeing in LY (3), SCs (2) and PM (1), but there was not have an AR signal in the germ cells (tailless arrows) that had staining by Scp3 (green) (× 400).

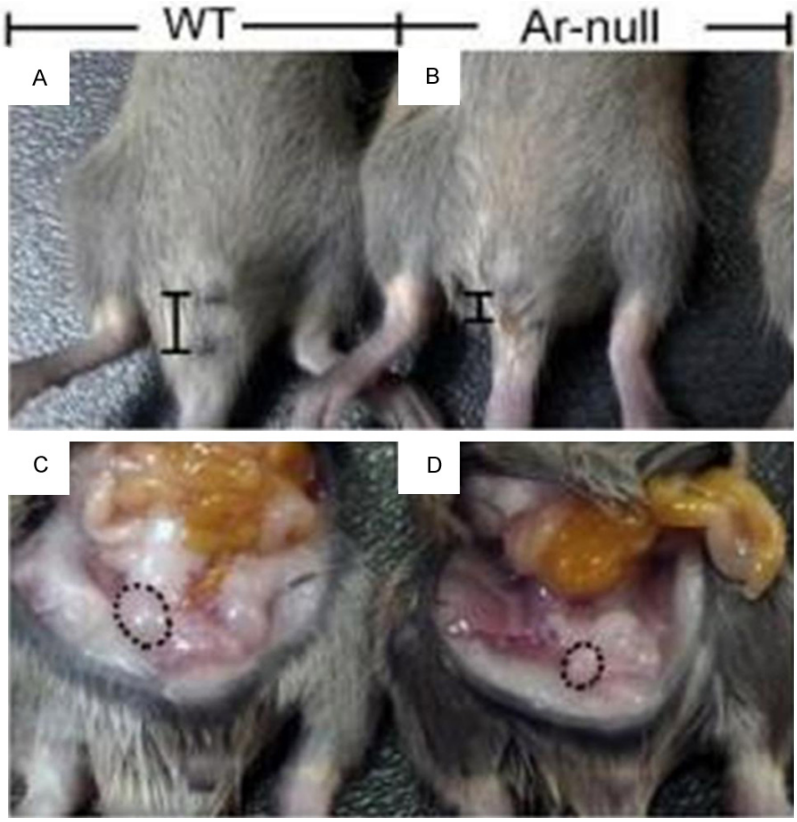

Figure 3. The male mice phenotypic characteristics after the birth of 21 days of age. (A, B) External genital organs in mice, Wild type mice (A), *Ar*-null mice (B). Internal genital organs in mice (C, D), Wild type mice (C), *Ar*-null mice (D).

software. Comparison between multiple sets of data using one-way variance statistical analysis. Statistical comparisons between the two groups were accomplished by Independent sample T-test. $P < 0.05$ considered statistically significant.

Results

Effect appraisal of the Ar gene knockout mice

The *Ar* knockout female mice mated with adult male mice C57BL/6J. Extracted the proteins from their offspring's tails. Then detect the proteins by the method of immunoblot (**Figure 1**), *Ar* gene was knockout (*Ar*-null). AR detected in WT while nothing detected in *Ar*-null, and this proved that *Ar* knockout is effective.

The expression of the Ar gene in testis

The wild type mice testicular tissue observed by using immunity fluorescence with 20 days of age; shows the AR have strong signals in three types of cells, such as the SCs, PM and LCs cells. However, there is no specific remarkable signal of AR protein in reproductive cells (**Figure 2**), which suggests that AR specifi-

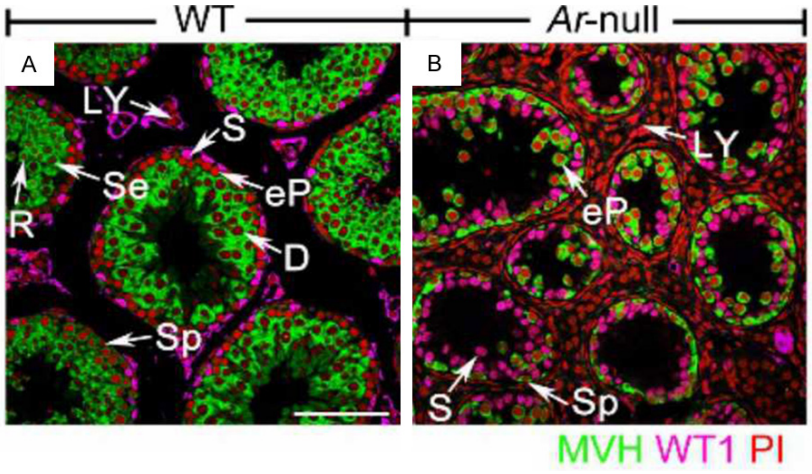

Figure 4. Testicular tissue of mice immunofluorescence after the birth of 21 days of age. MHV markers CGs (green), while WT1 markers SCs (pink). Wild type mice seminiferous tubules containing various levels sperm cells, such as spermatogonium (Sp), Early pachytene spermatocyte (eP), Diplotene spermatocyte (D) and Circular sperm (R) (A). There were only 2~3 layers of similar pachytene spermatocyte appeared in the Ar knockout mice seminiferous tubule (× 400) (B).

cally expressed in the SCs, PM and LCs in the cell of testicular tissue, with no expression in reproductive cells.

The phenotype changes of Ar gene knockout mice in reproductive system

The *Ar*-null mice's external genitalia obviously altered at the age of 21 in days. *Ar*-null mice appear the phenomenon of male feminization, the penis position change and perineum distance become shorter, similar with fake clitoris. The perineum distance changed from 17.6 mm to 7.3 mm. Dissection of the *Ar*-null mice and found the testicular volume decreased significantly (*P* value = 0.000) (**Figure 3**).

Seminiferous tubule cell phenotype of the Ar-null mice

The types, distribution and proportion of the seminiferous tubule cell have changed obviously in the *Ar*-null mice. The SCs, PM, and LCs of seminiferous tubule, the spermatogonium and each phase of the spermatocyte of the wild type showing the radial distribution. The SCs in the seminiferous tubule of *Ar*-null mice was abnormal, and some of them protruding from the base to the luminal. Spermatogonia were located at the base, with little similar pachytene spermatocytes, but there exists no haploid round spermatids (**Figure 4**). The results suggest that *Ar*-null mice spermatogenesis blocked during early in prophase I.

Homolog pairing blocked in Ar-null mice and spermatogenesis was arrest at the zygotene stage

Chromosome spreads showed that the percentage of spermatocytes in each stage of early meiosis I was significantly changed in the seminiferous tubules of *Ar*-null mice. Compared with wild-type mice, *Ar*-null mouse had a large number of the zygotene spermatocytes (over 70%) and its pachytene and diplotene spermatocytes significantly reduced. These results indicate that the spermatogenesis of *Ar*-null mice were arrest at the zygotene stage of meiosis I (**Figure 5A**). Testicular seminiferous tubal taken from 21-day-old *Ar*-null mice to do the immunofluorescence by using the centromere marker antiserum CREST. The synapsis of wild-type mice spermatocytes were completed from late zygotene stage to pachytene stage in meiosis I. At this time, there were 20 centromeres which represent there were 20 chromosomes. The chromosomes in *Ar*-null spermatocytes were not fully paired at late zygotene stage in meiosis I so that the number of centromere is more than 20 (**Figure 5B**).

Discussions

The Biological function of androgen mediated by specific androgen receptor (AR), to study the distribution of androgen receptors in the testicles has an important significance in understanding the ways and means to maintain and adjust sperm production process [11].

According to the results of our study, AR expressed in the Sertoli cells (SCs), Peritubular myoid cells (PM) and Leydig cells (LCs). That the AR being positive expressed in sertoli cells and myoid cells around the seminiferous tubule indicating the androgen participate in spermatogenesis. The AR specially expressed in the

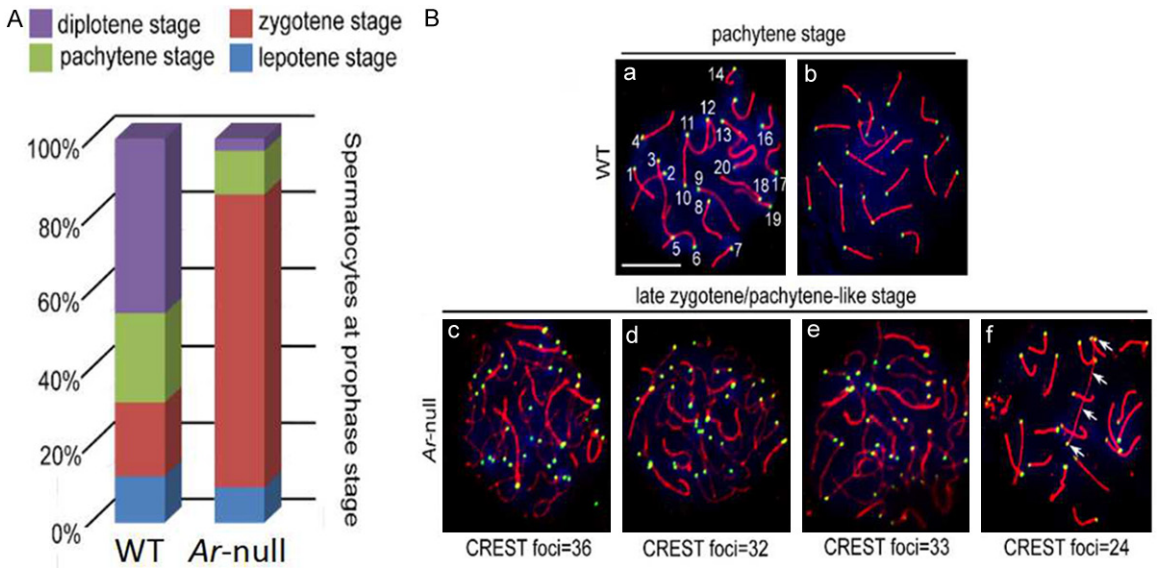

Figure 5. *Ar*-null mice spermatogenesis was arrest at the zygotene stage. (A) The percentage of wild type and *Ar*-null mice spermatogenesis in meiosis I at leptotene stage, zygotene stage, pachytene stage and diplotene stage. (B) Immunofluorescence of testicular seminiferous tubal which were taken from 21-day-old wild type and *Ar*-null mice. The centromere marker antiserum CREST is green and the antibody Scp3 is red. There were 20 CREST binding sites after the synapsis of pachytene stage in wild-type mice (a and b). There were more than 20 CREST binding sites at late zygotene and pachytene-like stage in *Ar*-null mice (c-f). The arrows in (f) indicated the chromatids, which appeared at pachytene-like stage in *Ar*-null mice. Scale bar: 5 μm.

sertoli cells suggests the sertoli cells specificity reaction of androgen on the process, and peritubular cells may be involved in maintain the process of sperm production [12]. However, the specific regulatory mechanism remains to further confirmed. Some researchers study with androgen receptor-deplete mouse and found that the fetal rat mesenchymal cells developed normally. As the growth of the age, it shown abnormal, which only has the partial function of natural mesenchymal cells of normal mice [13]. What is said above provides exquisite evidence that the androgen which mediated by AR are essential for mesenchymal cells development.

Spermatogenesis is dependent on androgen regulation and we have some controversy for whether AR expressed in germ cells (sperm cells) in previous studies. However, our experimental shows that *Ar* gene do not express in the germ cells.

In this study, we found the impacts of AR for the reproductive system by the *Ar* knockout mouse model .The systemic *Ar* knockout males comes up characteristics of the male feminization of the external. The main performance is the penis get shorter as the clitoris, and the perineum shortened, with a caecum vagina in. We can see the ectopic testis rise to abdomen after anatomical, its volume is smaller than the normal one. Spermatogenesis blocked in the early of the first reduction division and caused infertility. Some researchers believe that the loss of the gene AR is not the major reason that result in the stagnation of sperm, because by gene *Ar* knockout at the level of stem, the location of animal testicles descend revealed abnormal, which is similar to human undescended testicle. It is probably because that the testicles at higher temperature cause spermatogenesis stagnation [14]. Therefore, it is necessary to do experiments by gene AR specific knockout to explore the biological effect of the receptor. For example, we can selectively knock out the gene *Ar* of SCS, PM and LCS, and then observe the corresponding period that spermatogenesis are blocked. In this study, we found that the spermatogenesis was arrest at the zygotene stage because the homolog pairing blocked in *Ar*-null mice. However, why the homolog pairing was not happen is still unclear. We supposed that the reparation of double-strand breaks blocked at the zygotene stage so that the synapsis could not complete.

In recent years, the incidence of male infertility is increasing around the world according to the world health organization [15]. The results of in vitro show that the *Ar* gene closely related to spermatogenesis, the in-depth fundamental research about AR can give a diagnosis and treatment of male infertility with new perspective. Previous studies indicated that the serum testosterone level is differ with the levels that our body available to used, sperm production obstacle may be due to the AR changed [16], therefore, the detection of *Ar* gene polymorphism can be a diagnostic indicator to determine the cause of the infertility in clinical. Moreover, the patients with testicular biopsy that have been proven sperm production retardation can determine the stage of spermatogenesis block and the specific reasons through AR immunohistochemistry.

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Disclosure of conflict of interest

None.

Address correspondence to: Drs. Xiaoyan Wang and Changjun Zhang, Reproductive Medicine Center, Renmin Hospital, Hubei University of Medicine, No. 39, Chaoyang Road, Shiyan 442000, China. Tel: +86 13593741956; E-mail: dkeanig@163.com (XYW); changjunzhang@hotmail.com (CJZ)

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