

# Comparing Expression of Progesterone and Estrogen Receptors in Testicular Tissue From Men With Obstructive and Nonobstructive Azoospermia

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**ABSTRACT:** The objective of this study was to identify and compare the expression profiles of progesterone receptor (PR) and estrogen receptor  $\alpha$  (ER $\alpha$ ) in the testes of men with obstructive azoospermia (OA), maturation arrest (MA), and Sertoli cell–only (SCO) histology. Testicular biopsies were obtained from 10 patients with OA, 10 patients with MA (either early or late arrest), and 8 patients with SCO who did not have hormonal abnormalities and varicoceles. Expression of PR and ER $\alpha$  was detected by immunofluorescence and Western blot. PR was expressed in the spermatogenic, Leydig, and Sertoli cells in the testes of OA patients. In the MA and SCO patients, the expression of PR was reduced in all cell types as compared with that in the OA patients. Western blot demonstrated that both the full-size (120 KDa) and the truncated (52 KDa) isoforms of the PR were expressed in the OA and MA testes. However, in the

SCO testes, only the truncated isoform of PR (52 KDa) was expressed. ER $\alpha$  (66 KDa) was expressed principally in the spermatogenic and Leydig cells in the OA testes. By immunohistochemistry staining, expression of ER $\alpha$  was decreased in the spermatogenic and Leydig cells of the MA testes, whereas its expression was enhanced in the Leydig cells of the SCO testes. However, by Western blot, expression of ER $\alpha$  was significantly reduced in the SCO testes as compared with that in the OA and MA testes. We conclude that PR and ER $\alpha$  may play a role in the pathogenesis of the MA and SCO phenotype in patients with infertility.

Key words: Estrogen receptor  $\alpha$ , male infertility.

**J Androl 2009;30:127–133**

Though regarded as principally female hormones, progesterone and estrogen play important roles in male reproduction. Progesterone has been found to influence spermatogenesis, Sertoli cell function, spermiogenesis, sperm capacitation, and testosterone biosynthesis (Sauber et al, 1996; El-Hefnawy et al, 2000; Schwarzenbach et al, 2003; Oettel and Mukhopadhyay, 2004). Estrogen has been found to affect proliferation and differentiation of gonocytes and spermatogonia and to inhibit apoptosis of the postmeiotic spermatogenic cells (O'Donnell et al, 2001; Hess and Carnes, 2004). In vertebrates and humans, the biological activity of progesterone and estrogen is mediated by progesterone receptor (PR) and estrogen receptor (ER) respectively. PR

and ER share similar structural and functional characteristics and have highly conserved structural domains involved in DNA and ligand binding (O'Donnell et al, 2001). The signaling mechanisms underlying the action of PR and ER have been extensively investigated. Two kinds of actions of this superfamily of receptors have been discovered so far. Receptors for classic genomic actions generally reside in the nucleus and/or in the cytoplasm. In contrast, receptors for the nongenomic (also called nonclassic) actions reside on the cell surface (Revelli et al, 1998). The classic action includes a conformational change in the receptor, translocation of the ligand complex to the nucleus where it interacts with DNA binding elements in the genome, and alterations of the transcription of the steroid responsive genes. The rapid nongenomic mode of action does not involve transcriptional process, but includes activation of sarcoma/extracellular signal-related kinase signaling pathways, stimulating calcium influx, tyrosine phosphorylation of proteins, chloride efflux, and increasing in cyclic adenosine monophosphate in many tissues including the testis and spermatozoa (Gadkar-Sable et al, 2005). Recently, progesterone and estrogen have been discovered to take part

Supported in part by grants from the American Foundation for Urologic Disease (H.L.F.) and the scientific research grant of the Chinese University of Hong Kong (c001-2041283) (Y.B.H.).

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Received for publication March 3, 2008; accepted for publication September 4, 2008.

DOI: 10.2164/jandrol.108.005157

in both the nongenomic signaling pathways, which cause rapid effects in different cell types including spermatozoa and the classic actions (Luconi et al, 2004).

The classic PR-A (90 KDa) and PR-B (120 KDa) and the membrane-bound PR (55 KDa) have all been detected in the human testes previously (Shah et al, 2005). The stage-specific expression of PR is most restricted to the spermatogenic cells and Sertoli cells in the testis (Shah et al, 2005). The mice null for steroidal receptor coactivator-1 (SRC-1; an intracellular PR coactivator) show reduced testicular growth and fertility as compared with wild-type littermates (Xu et al, 1998). Two kinds of ER (ER $\alpha$  and ER $\beta$ ) have been discovered so far in the human male reproductive tract. ER $\beta$  locates mainly in somatic cells and/or primary spermatocytes. Its polymorphism is associated with male infertility (Aschim et al, 2004, 2005). In contrast, ER $\alpha$  mainly locates in the efferent ductule epithelium and also in the Sertoli and Leydig cells in human testes (Pelletier and El-alfy, 2000; Taylor and Al-Azzawi, 2000; Makinen et al, 2001). However, some studies show no ER $\alpha$  expression in human testes (Makinen et al, 2001). Two ER $\alpha$  isoforms (66 and 46 KDa) have been demonstrated in rat testes and human immature germ cells (Lambard et al, 2004; Staub et al, 2005). Importantly, the polymorphism of ER $\alpha$  is associated with elevated plasma levels of follicle-stimulating hormone (FSH) and idiopathic azoospermia in humans (Suzuki et al, 2002). The commonly accepted role of ER $\alpha$  is to reabsorb the fluid of the seminiferous tubules just before the spermatozoa enter into the epididymis, which might concern the function of aquaporins (Picciarelli-Lima et al, 2006).

It is still unknown whether there are any differences in the expression of PR and ER $\alpha$  between normal and infertile human testes. The objective of this study is to determine the expression of PR and ER $\alpha$  in the testes of men with infertility, and the possible relationship with the pathogenesis of this condition. It is the first report of abnormal expression of both PR and ER $\alpha$  in the testes of infertile men.

## Materials and Methods

### Tissue Preparation

Twenty-eight specimens were obtained from fresh biopsy and archival tissue blocks from men undergoing biopsies for either azoospermia or severe oligozoospermia. The use of these specimens for the present study was approved by the University of Iowa and Chinese University of Hong Kong Institutional Review Board (IRA). The specimens demonstrated either normal spermatogenesis with obstructive azoospermia (OA; n = 10), maturation arrest (MA; early or late; n = 10) or Sertoli cell-only (SCO; n = 8) histology. The ages of the patients ranged from 23 to 42 years. None of the men biopsied had hormonal abnormalities

or varicoceles. Fresh specimens were fixed in Carnoy solution and embedded in paraffin. Archival tissues were fixed in Bouin solution and embedded in paraffin. All sections were cut at 5  $\mu$ m, mounted, and deparaffinized, then stained using indirect immunofluorescence techniques as described below.

### Indirect Immunofluorescence Techniques

The primary antibodies for PR and ER $\alpha$  were polyclonal rabbit anti-human PR IgG (Cat SC-539; Santa Cruz Biotech, Santa Cruz, California), which was formed against the internal region, and polyclonal mouse anti-human ER $\alpha$  IgG (Cat SC-8002; Santa Cruz Biotech), which was against the C terminus. Tissue specimens were incubated with the primary PR and ER $\alpha$  antibodies at a 1:100 dilution overnight at room temperature. All incubations were carried out in a humidified chamber. Sections were rinsed 5 times with diluent and incubated for approximately 45–60 minutes with 1:50 dilution of fluorescein-conjugated affinity-purified goat antiserum to rabbit IgG (Santa Cruz Biotech). The sections were then washed 5 times with diluent and viewed by epifluorescence with a Zeiss IM 35 microscope (Oberkochen, Germany). Tissue sections incubated with either buffer, normal serum (normal rabbit serum; Sigma, St Louis, Missouri), or the secondary conjugated antibody without primary antibody were utilized as negative controls. Staining was considered positive if the tissue demonstrated immunofluorescence staining, and was graded on a relative scale of no staining, weak staining, and prominent staining.

### Western Blot Analysis

Tissue specimens were homogenized in Tris-HCl with 1 mM serine protease inhibitor PMSF. The homogenates were centrifuged at 11 950  $\times$  g for 10 minutes to remove cellular debris. Proteins were extracted with 1% sodium dodecyl sulfate (SDS) for 2 hours at 4°C. Protein concentrations of the extracts were determined from the absorbency at 260 and 280 nm by spectrophotometer. Aliquots (30–50  $\mu$ g) of the protein extracts were separated by electrophoresis on a 12% SDS-polyacrylamide gel electrophoresis gel, transferred to nitrocellulose paper, and blocked with 5% milk powder in TBS buffer (10 mM/L Tris-HCl, pH 8.0, 150 mM/L NaCl) for 1 hour at room temperature. The blots were washed in TBST (TBS containing 0.05% Tween-20) and then incubated overnight with PR and ER $\alpha$  polyclonal antibody diluted to 1:400 with phosphate-buffered saline containing 2.5% bovine serum albumin. The blots were then washed in TBST, incubated for 1 hour with horseradish peroxidase-conjugated anti-rabbit immunoglobulins (Cappel, Durham, North Carolina) diluted to 1:10 000 in TBST and followed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, Illinois) staining. This experiment was repeated 3 times using separate samples.

## Results

### PR Studies

PR was found to stain strongly in the cytoplasm of the spermatogonia, spermatogenic cells, Leydig cells, and

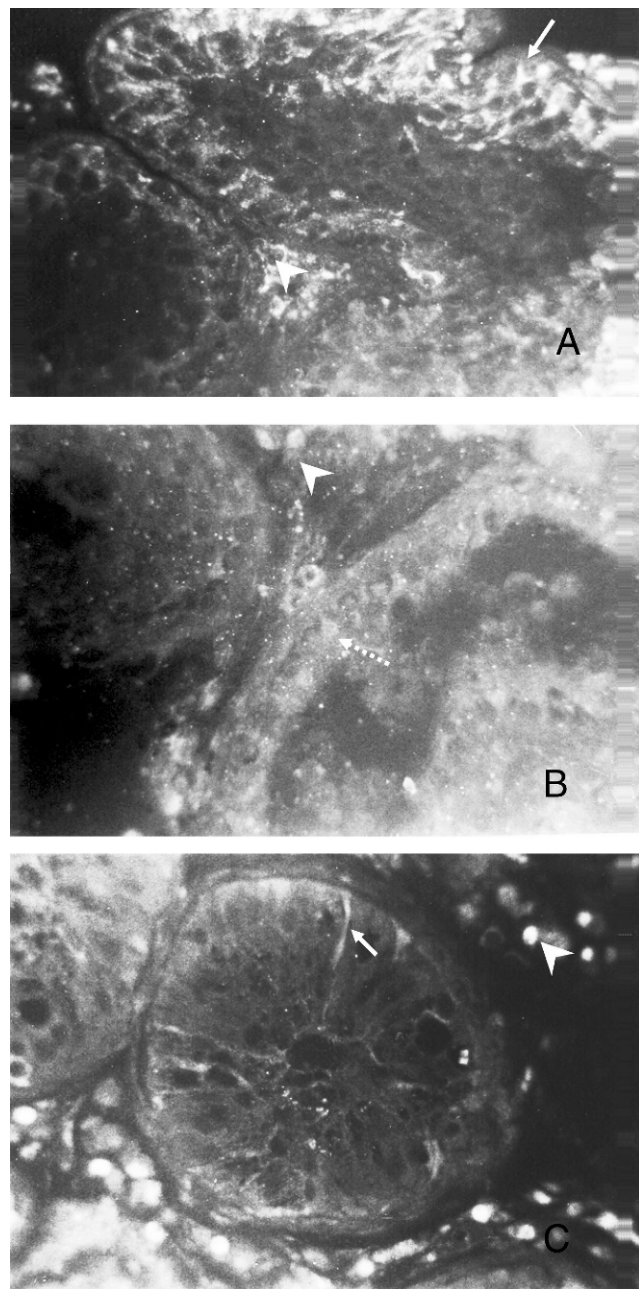


Figure 1. Immunofluorescent staining of progesterone receptor (PR) in human testes. PR is stained strongly in the spermatogonia, spermatogenic cells, Leydig cells, and Sertoli cells in the obstructive azoospermia testes (A). In the maturation arrest testes, PR is stained weakly in the spermatogonia, spermatogenic cells, and Leydig cells (B). PR is stained weakly in the Sertoli cells and strongly in the Leydig cells in the Sertoli cell-only testes (C). Sertoli cells are indicated by solid arrows. Leydig cells are indicated by arrow heads. Spermatogenic cells are indicated by dashed arrows.

Sertoli cells in the OA testes (Figure 1A). In the MA testes tissues, PR was found to stain weakly in the spermatogonia, spermatogenic cells, and Leydig cells (Figure 1B). PR also stained weakly in the Sertoli cells and strongly in the Leydig cells of the SCO testes tissues

(Figure 1C). Negative controls exhibited no staining in each case. It should be noted that expression of PR in the Leydig cells of the SCO testes was restricted to the nuclei (Figure 1C). The Western blot showed that different isoforms of PR (52 and 120 kDa) existed in the OA and MA testes tissues (Figure 2A, lanes NL and MA). However, in the SCO testes tissue, only the 52-KDa isoform was present (Figure 2A, lane S).

#### *ER $\alpha$ studies*

ER $\alpha$  was strongly localized in the nuclei of the spermatogenic cells and the cytoplasm of the Leydig cells in the OA testes (Figure 3A), and was weakly expressed in the MA testes (Figure 3B). In the SCO testes, expression in Leydig cells was significantly increased, whereas the Sertoli cells were negative for ER $\alpha$  staining (Figure 3C). Negative controls exhibited no staining in each case (Figure 3D). Western blot data demonstrated that both OA and MA testes had high expression of ER $\alpha$  at 66 kDa (Figure 2B, lanes NL and MA); however, the expression in SCO testes was dramatically reduced (Figure 2B, lane S).

## **Discussion**

### *Different Isoforms of PR/ER $\alpha$ Expressed in Human Testes*

The PR gene is differentially transcribed into several isoforms, including the genomic (PR-A, PR-B, PR-C, PR-S, and PR-T) and the membrane-bound (mPR $\alpha$ , mPR $\beta$ , and mPR $\gamma$ ). PR-A and PR-B are translated from the same gene; however, their transcription is initiated at different promoters. The PR-A isoform differs from the PR-B isoform in lacking the first 164 amino acids contained in PR-B (Hanekamp et al, 2003). PR-C is smaller than the other 2 isoforms, truncated at the N-terminal domain, and known to have unique transcriptional potentiating properties (Wei et al, 1997). Other alternative transcriptions of PR gene generate the other isoforms of PR, such as PR-S and PR-T. Multiple PR mRNA transcripts have been shown to exist in a variety of human tissues, which give rise to several molecular weights (eg, 120, 90, 76–82, and 60–64 KDa; Gadkar-Sable et al, 2005). It has been found that both the genomic (90 and 120 KDa) and the membrane-bound PRs (55 and 29 KDa) exist in the human testes (Sauber et al, 1996; Luconi et al, 1998, 2004; Losel et al, 2005; Shah et al, 2005). As a regulator of reproductive events, progesterone has been detected at high levels in the testicular tissue (Leinonen et al, 1980; Gadkar-Sable et al, 2005). Following binding to its intracellular and membrane receptor, progesterone begins to act in both a genomic mode and a nongenomic mode.

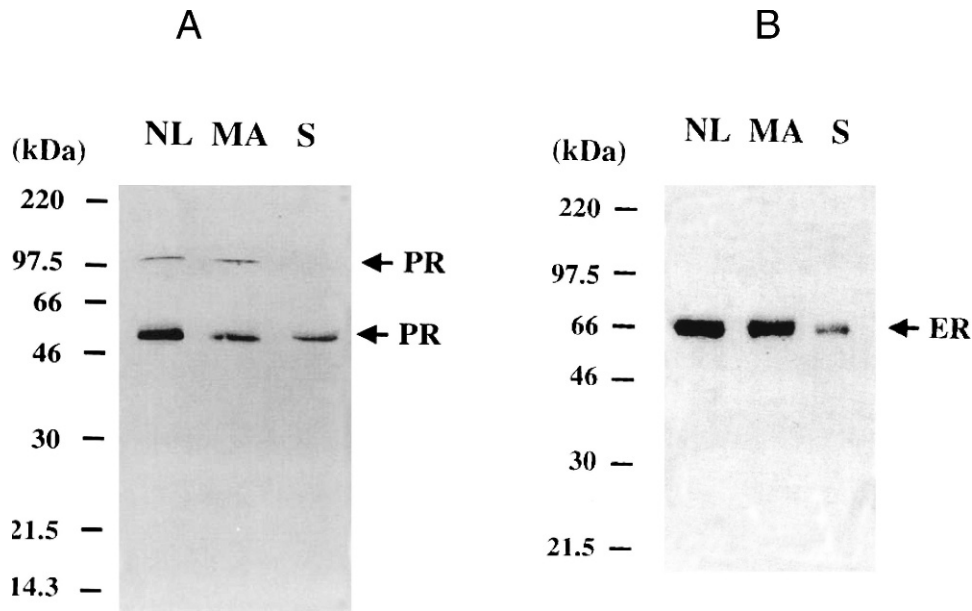


Figure 2. Western blot analysis of progesterone receptor (PR) and estrogen receptor  $\alpha$  (ER $\alpha$ ) in human testes. The truncated (52 KDa) and full-size (120 KDa) isoforms of PR are expressed in the obstructive azoospermia (OA) and maturation arrest (MA) testes (A, lanes NL and MA). However, only the truncated isoform is expressed in the Sertoli cell-only (SCO) testes (A, lane S). ER $\alpha$  expressed in the OA and MA testes (B, lanes NL and MA) is much more than that expressed in the SCO testes (B, lane S).

In this study, 2 isoforms of PR (120 and 52 KDa) are identified in human testes, which might be the classic and the nonclassic membrane-bound respectively. Expression of the 120-KDa isoform of PR is absent in the testes of SCO patients, and the expression of the 52-KDa isoform of PR is decreased in the SCO and MA testes according to the Western blot (Figure 2A). This may indicate that: 1) Expression of the 52-KDa isoform of PR in the OA testes is significantly higher than that in the MA and SCO testes. Thus, the 52-KDa isoform of PR might be independently transcribed upon the full-length isoform. 2) Expression of PR in the Sertoli cells is decreased in the MA testes and almost no PR is detected in the SCO testes. So, in contrast to the expression of PR mRNA, which is restricted to the spermatogenic and Sertoli cells as previously indicated, we postulate that the expression of the 120-KDa isoform of PR is mostly restricted to the spermatogenic cells (Shah et al, 2005). 3) Expression of PR in the Leydig cells is enhanced in the SCO testes. Because the expression of PR mRNA is only occasionally detected in the Leydig cells in normal human testes, we hypothesize that the increased expression of PR in the Leydig cells of the SCO patients might indicate a different type of PR or a potentially different control system for PR in the spermatogenic cells and in the Leydig cells (Shah et al, 2005). This is consistent with the conclusion that the PR (45–57 KDa) expressed in the murine Leydig cell line is the nonclassic type (El-Hefnaway et al, 2000). In this study, we found that the PR is expressed in the

cytoplasm of the spermatogenic and Sertoli cells and the nuclei of the Leydig cells. Furthermore, the expression of the 52-KDa PR in the Leydig cells is significantly increased in the SCO testes based upon histological staining (Figure 1). We conclude that the absence of the classic type of PR in the spermatogenic cells might be involved in the spermatogenic failure seen in the MA and SCO testes. The absence of the classic PR in the spermatogenic cells might stimulate the expression of the nonclassic PR in the Leydig cells possibly through a negative feedback mechanism.

Two forms of ER (ER $\alpha$  and ER $\beta$ ) have been discovered so far. Expression of ER $\beta$  is more ubiquitous than that of ER $\alpha$  (Taylor and Al-Azzawi, 2000). Expression sites of ER $\alpha$  in the testes are controversial. Some authors have detected ER $\alpha$  in the nuclei of the Leydig cells in humans, whereas others indicate that ER $\alpha$  is expressed in the Sertoli, Leydig, and peritubular myoid cells in the rat and is restricted to the Leydig cells in the fetal testes (Pelletier and El-alfy, 2000; Makinen et al, 2001; O'Donnell et al, 2001; Lucas et al, 2008). Two isoforms of ER $\alpha$  (66 and 46 KDa) have been found in the human and primate testes because of alternative splicing of the C-terminal region (Saunders et al, 2002; Hirata et al, 2003; Staub et al, 2005). The nonclassic ER $\alpha$  (67 KDa) is expressed in human ejaculated spermatozoa and directly interacts with the phosphatidylinositol-3-OH kinase pathway. Variant types of ER $\alpha$  (66 and 46 KDa) have been detected in human

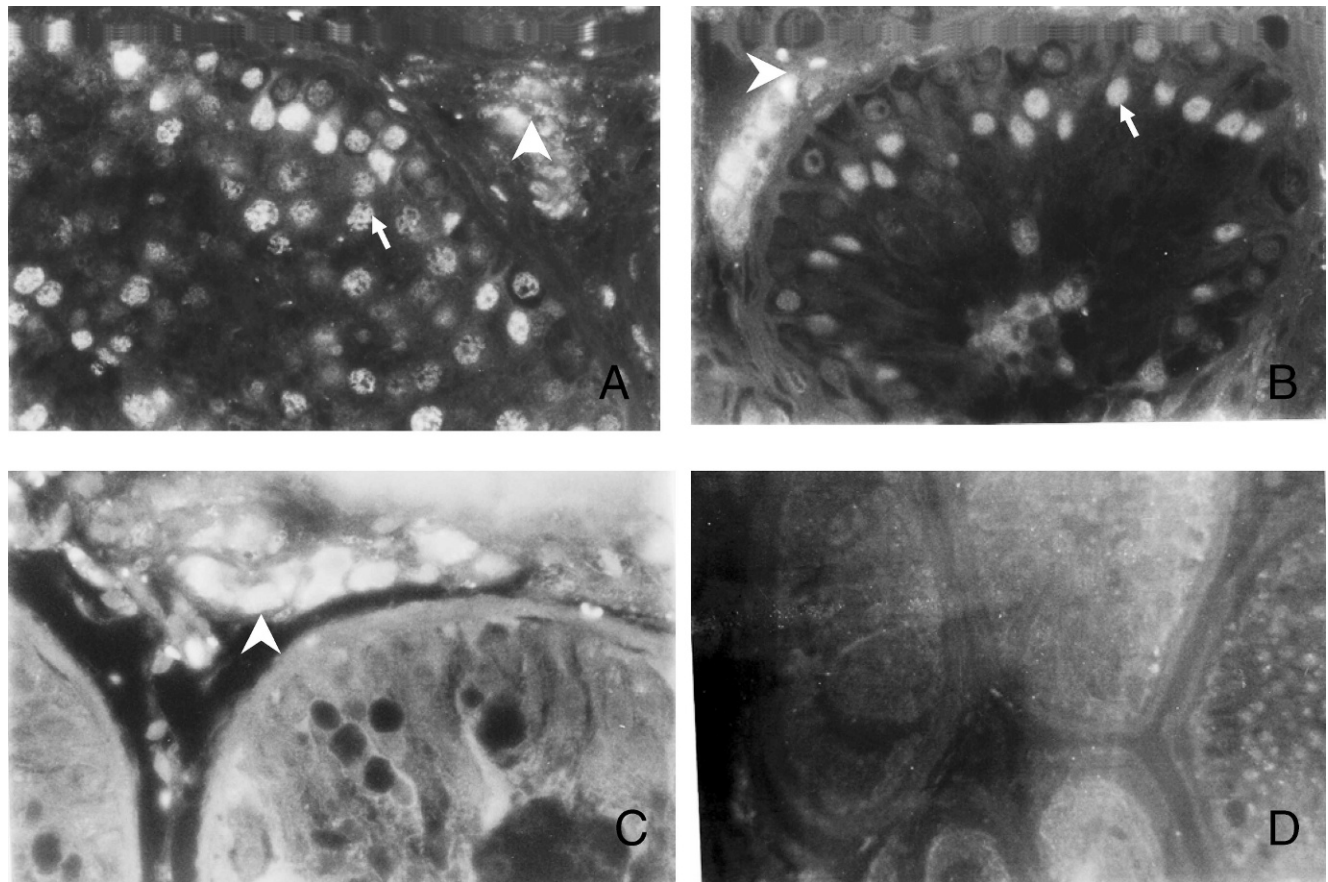


Figure 3. Immunofluorescent staining of estrogen receptor  $\alpha$  ( $ER\alpha$ ) in human testes.  $ER\alpha$  is strongly stained in the spermatogenic and the Leydig cells in the obstructive azoospermia testes (A), and is weakly stained in the maturation arrest testes (B).  $ER\alpha$  is not expressed in the Sertoli cells but is expressed in the Leydig cells in the Sertoli cell-only testes (C). (D) is the negative control. Sertoli cells are indicated by solid arrows. Leydig cells are indicated by arrowheads.

spermatogenic cells, whereas only the 66-KDa isoform of  $ER\alpha$  is detected in human mature spermatozoa (Lambard et al, 2004). In this study, only the 66-KDa  $ER\alpha$  expressed in the nuclei of the spermatogenic cells and the cytoplasm of the Leydig cells is detected. Expression of  $ER\alpha$  in the spermatogenic cells decreases in the MA testes by immunofluorescence staining, whereas the expression is similar to Western blot (Figures 2B and 3). The Sertoli cells are negative for  $ER\alpha$ . We found that although the expression of  $ER\alpha$  is enhanced in the Leydig cells in the SCO testes, its expression in the whole testes is dramatically decreased as indicated by Western blot. We conclude that: 1) expression of  $ER\alpha$  in the spermatogenic cells might be important for normal spermatogenesis; 2) the patterns of expression in the spermatogenic cells (nucleus pattern) and the Leydig cells (cytoplasm pattern) might indicate that different types of  $ER\alpha$  exist in the 2 cell types, though only 1 isoform (66 KDa) is discovered here in human testes.

#### *Functional Analysis of the $PRIER\alpha$ Expressed in Human Testes*

Direct functions of progesterone in testes include steroidogenesis, Sertoli cell function, and spermatogenesis (Walton et al, 2006). Progesterone and its analogues (including  $17\alpha$ ,  $20\beta$ -dihydroxy-4-pregnen-3-one and  $17\alpha$ ,  $20\alpha$ -dihydroxy-4-pregnen-3-one) are reported to induce hyperactive motility, acrosomal reaction, spermatogonial DNA synthesis, and spermatogenic meiosis in mammals, fish, and amphibians (Calogero et al, 2000; Losel and Wehling, 2003; Losel et al, 2003; Miura et al, 2006). Furthermore, the expression of PR on the spermatozoa has been shown to be related to sperm fertilizing capability in humans (Fukui et al, 2000). However, in contrast to adult female mice with disrupted classic PR, which display significant defects in reproductive functions, adult male mice with disrupted classic PR show no impairment of fertility (Dube and Tremblay, 1979; Heikinheimo et al, 1995; Lydon et al, 1995).

Being produced within the testes by the aromatization of testosterone, estrogen disrupts the development of fetal Leydig cells, inhibits testosterone production in the Leydig cells, stimulates gonocyte and spermatogonia proliferation, and enhances spermatogenesis by inhibiting apoptosis of the postmeiotic spermatogenic cells (Kular, 1988; Li et al, 1997; Miura et al, 1999). Estradiol has been confirmed to exert stimulatory effects on premitotic DNA synthesis in the stage I segment of the seminiferous epithelium in vitro and to function directly on neonatal rat testicular gonocytes by the PDGF or HSP90 pathways (Thuillier et al, 2003; Wang et al, 2004; Wahlgren et al, 2008). The inhibitory effects of estrogens on testicular steroidogenesis are mediated exclusively via ER $\alpha$  (Cederroth et al, 2007). As a result, in ER $\alpha^{-/-}$  mice, plasma/testicular testosterone concentrations, spermatogenesis, steroidogenesis, and fertility are influenced (Walker and Korach 2004; Gould et al, 2007). In ER $\alpha^{-/-}$  mice, Leydig cell volumes are decreased and a significant loss of all kinds of spermatogenic cells through active caspase-3 activation and infertility occurs (Gould et al, 2007). However, the spermatogenic cells of ER $\alpha^{-/-}$  mice have normal functions after transplantation to wild-type mouse testes (Mahato et al, 2000). Estrogen also affects Sertoli cell proliferation and may suppress differentiation, but no changes were observed on the number of Sertoli cells and spermatogonia in the ER $\alpha^{-/-}$  mouse. So, the function of ER $\alpha$  on germ cells remains questionable as revealed in this mouse model. Similarly, in human patients with abnormalities of ER $\alpha$  and aromatase genes, high levels of testosterone, luteinizing hormone (LH), and FSH are displayed. These men also have low sperm viability, number, and motility; azoospermia; and sometimes cryptorchidism (Smith et al, 1994; Herrmann et al, 2002; Suzuki et al, 2002; Maffei et al, 2004; Guarducci et al, 2006).

In this study, testicular tissue from OA and nonobstructive azoospermia (NOA) patients, including MA and SCO, is used. MA is characterized by the presence of germ cells that do not complete spermatogenic development, as seen on testicular biopsy. SCO is characterized by the presence of no germ cells but only Sertoli cells as seen on testicular biopsy. Both groups of NOA patients in this study did not show an increase in serum FSH. However, it is unclear whether progesterone, estrogen, and/or testosterone changes in these patients. Disruption of PR and ER in the testes might cause the steroid hormones mentioned above to change accordingly. It has been demonstrated that progesterone stimulates testosterone synthesis in the rat testes by regulating the steroid acute regulatory gene (StAR; Schwarzenbach et al, 2003). And increased testosterone, LH, and FSH synthesis might also occur as a result of ER $\alpha$  mutations via the negative effect on the inhibition

of testicular steroidogenesis (Cederroth et al, 2007). Therefore, the combined decrease of PR and ER $\alpha$  might be one of the major causes for the infertility of the MA and SCO patients.

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