Differential Expression of Estrogen Receptors (ERα/ERβ) in Testis of Mature and Immature Pigs

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ABSTRACT

High affinity estrogen receptors (ERs) mediate estrogen action in male reproductive tissues. The objective of the present study was the immunolocalization of estrogen receptor α and estrogen receptor β in immature and mature testes of pig, a species in which the role of estrogens on gonadal function is scarcely known. Testes from 3 and 18 month-old pigs were investigated. Immunohistochemistry was performed on paraffin embedded-tissues using both mouse anti-human monoclonal IgG ERα and IgG ERβ 1 isoform. Western blot analysis demonstrated antibody specificity. ERα staining was not observed in immature testes, but it was detected in spermatogonia, spermatocytes and in the most Leydig cells of mature testes. ERβ immunoreactivity was observed in spermatogonia and Leydig cells of immature gonads, while it was clearly detected in spermatogonia and in spermatocytes of adult pig testes. The differential ERα/ERβ expression in germ and somatic cells of the gonads suggest a role of estrogens in function and in development of pig testis. © 2004 Wiley-Liss, Inc.

Key words: estrogens; estrogen receptors; testis; pig development

It is well known that estrogens play a pivotal role in female development and reproduction, but a growing body of evidence indicates they are also involved in the physiology of male genital system (Hess, 2003). Estrogen action is mediated by estrogen receptor proteins (ERs), which are expressed in the male reproductive tract (O'Donnell et al., 2001; Carreau et al., 2003), although their biological significance is not well defined yet.

ERs occur in two forms, the classical ERα subtype and the novel ERβ subtype subsequently discovered in rat (Kuiper et al., 1996), human (Mosselman et al., 1996; Ogawa et al., 1998), and mouse (Tremblay et al., 1997). Both subtypes have been detected in testes and/or epididymis of different mammals such as the rat (Hess et al., 1997), mouse (Zhou et al., 2002), human (Ergun et al., 1997; Makinen et al., 2001; Saunders et al., 2001), dog and cat (Nie et al., 2002), but their cellular expression often varied across the species. Furthermore, a differential ERβ and/or ERα cellular expression has been reported during testis ontogeny in rat and mouse (van Pelt et al., 1999; Jefferson et al., 2000), indicating a possible involvement of these receptors in gonadal development.

In the past years, relatively few investigations have addressed the role of estrogens in pig male development. Reports have described the aromatase localization in Leydig cells of porcine testes (Fraczek et al., 2001) and the ERα expression in the efferent ducts of the newborn piglets (Nielsen et al., 2001). Furthermore, recently we dem-

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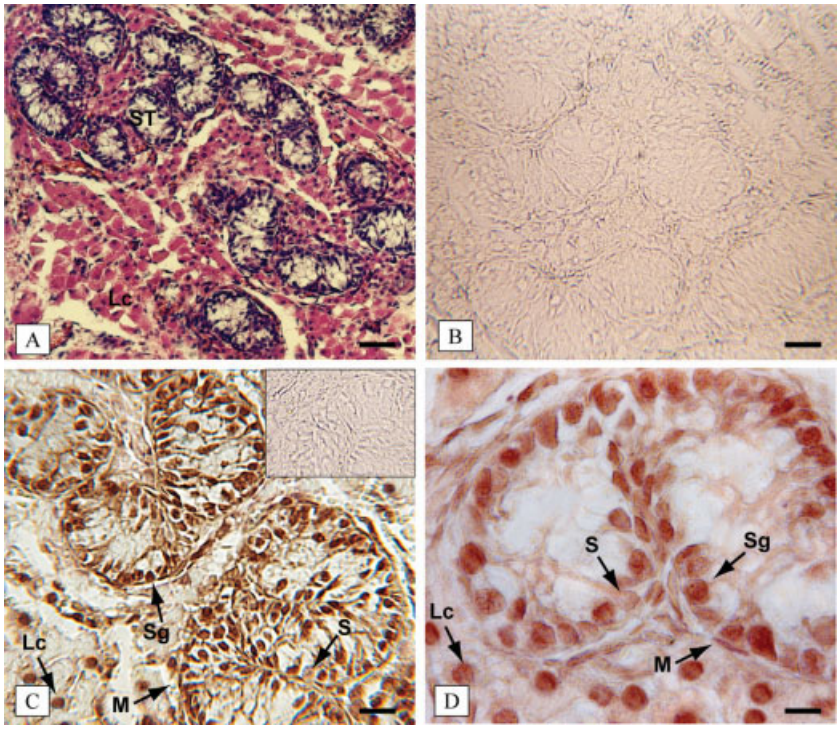

Fig. 1. Morphology and ERα/ERβ immunoreactivity of immature pig testes. **A:** Haematoxylin-eosin staining. **B:** ERα negative immunostaining. **C** and **D:** ERβ expression pattern. Insert: Absorption control. Scale bars = 25 μm (A, B); 12.5 μm (C); 5 μm (D). ST, seminiferous tubule; Lc, Leydig cells; S, Sertoli cells; Sg, spermatogonia; M, peritubular myoid cells.

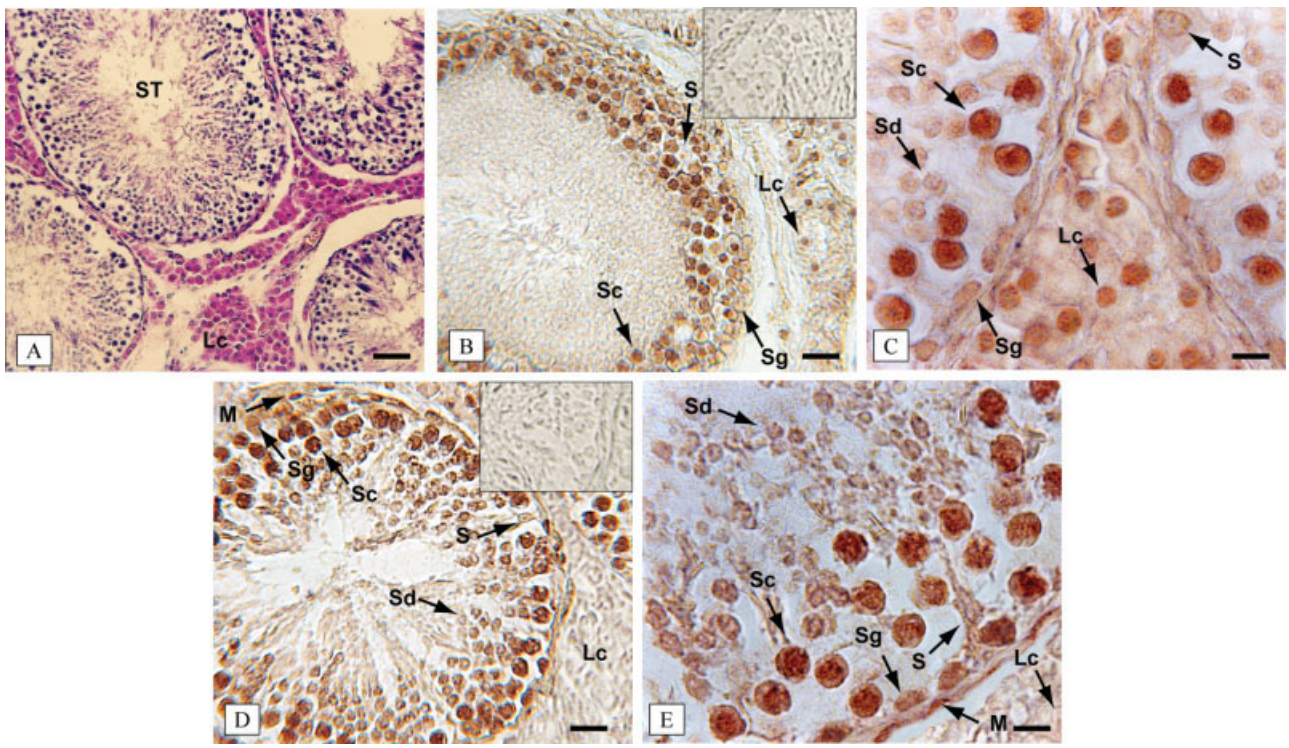

Fig. 2. Morphology and ERα/ERβ immunoreactivity of mature pig testes. **A:** Haematoxylin-eosin staining. **B** and **C:** ERα expression pattern. **D** and **E:** ERβ expression pattern. Inserts: Absorption controls. Scale bars = 25 μm (A); 12.5 μm (B, D); 5 μm (C, E). ST, seminiferous tubule; Lc, Leydig cells; S, Sertoli cells; Sg, spermatogonia; Sc, spermatocytes; Sd, spermatids; M, peritubular myoid cells.

onstrated the ERβ distribution in pig epididymis (Carpino et al., 2004), but the expression pattern of the two ER subtypes in pig testes is still unknown. Therefore, in the present study, the immunohistochemical localization of ERs was studied in immature and mature testes of *Sus scrofa domestica* using antibodies to ERα/ERβ and Western blot analysis to demonstrate antibody specificity.

MATERIALS AND METHODS

Animals and Testis Sections

Testes were removed from four immature (3-month-old) and four mature (18-month-old) pigs (*Sus scrofa domestica*) during routine castrations at the local animal hospital. Tissues were immediately fixed in neutral buffered formalin (4%), dehydrated in a series of ethanol concentrations, and paraffin-embedded. Then testis sections (5 μm) were cut (8–9 serial sections for each sample), mounted on polylysine-precoated slides, deparaffinized, and rehydrated. Morphological analysis was carried out by standard haematoxylin-eosin staining.

Immunohistochemistry

Immunostaining was performed after heat-mediated antigen retrieval (sections microwaved in a 0.01 M citrate buffer solution, pH 6, for 18 min). Hydrogen peroxide (3% in distilled water for 30 min) was used to inhibit endogenous peroxidase activity. Normal goat serum (10% for 30 min) was used to block nonspecific binding sites. Immunodetection of estrogen receptors was performed using both mouse antihuman monoclonal IgG ERα (F10; 1:40; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse antihuman monoclonal IgG ERβ 1 isoform (MCA1974; 1:60; Serotec, Oxford, U.K.) as primary antibodies (overnight at 4°C). A biotinylated goat antimouse IgG (Santa Cruz Biotechnology) was utilized as secondary antibody (1 hr at RT) for both the ERs. Avidin-biotin-horseradish peroxidase complex (Santa Cruz Biotechnology) amplification was then performed (30 min at RT) and the peroxidase reaction was developed with diaminobenzidine (Stable DAB, Sigma Chemical, Italy). The primary antibody was replaced by normal mouse serum in the ordinary control. In addition, absorption control was assessed by using a primary antibody preabsorbed with an excess of the specific purified proteins (both ERα and ERβ; PanVera, Invitrogen, Milan, Italy) for 48 hr at 4°C.

HeLa cells, which do not express estrogen receptors, were used as negative control. The same cell line, transiently transfected with plasmids encoding both ERα and ERβ, provided the positive controls.

Cell Culture, Plasmids, and Transfections

HeLa cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air and were cultured in DMEM (Sigma-Aldrich, Milan, Italy) without phenol red supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 U/ml), and 10% fetal bovine serum (Life Technology, Milan, Italy). ERα, cloned into pSG5 (Tora et al., 1989), and ERβ, cloned into pCMV5 (Kuiper et al., 1996), were a gift from Didier Picard (Geneve, Switzerland). ERα and ERβ expression plasmids (5 μg) were transfected for 6 hr (100 mm dishes) in medium without serum using the Fugene6 Reagent as recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany).

TABLE 1. Immunostaining for estrogen receptors in immature and mature pig testes*

	ERα		ERβ	
	Immature	Mature	Immature	Mature
Leydig cells	−	++	+++	−
Peritubular myoid cells	−	−	+++	+++
Sertoli cells	−	−	−/+	−
Spermatogonia	−	+	+++	++/+++
Spermatocytes	≡	+++	≡	+++
Spermatids	≡	−	≡	−

*Staining intensity scores were as follows: −, negative; +, weak staining; ++, moderate staining; +++, strong staining; ≡, cell type absence.

Western Blot Analysis

Frozen samples of immature and mature pig testes were homogenized (GLAS-COL, Terre Haute) and lysed in buffer containing 20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.2 mM EDTA, 25% glycerol, 1 mM 1,4-dithio-threitol, 0.5 mM, Na_3VO_4, 0.2% Nonidet P-40, and a mixture of protease inhibitors (aprotinin, leupeptin, phenylmethylsulfonylfluoride, pepstatin). Lysates were quantified using Bradford protein assay reagent (Sigma-Aldrich) and equal amounts of proteins (40 μg) were resolved on a 10% sodium dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in glycine buffer (0.02 mM Tris, 0.2 mM glycine, 1% SDS). Proteins were then transferred to a nitrocellulose membrane (Amersham, Milan, Italy) and probed overnight at 4°C with antibodies against ERα (F10; Santa Cruz Biotechnology), ERβ (MCA1974; Serotec), and β-actin (Serotec). Finally, proteins were revealed using the ECL system (Amersham). The experiments were repeated 10 times.

RESULTS

Morphological Analysis

Immature pig testes were characterized by the presence of small closed seminiferous tubules with an epithelium containing Sertoli cells and spermatogonia (Fig. 1A). Furthermore, peritubular myoid cells, blood vessels, and clusters of Leydig cells were present in the interstitium.

The testes of mature pigs revealed highly developed seminiferous tubules with a wide lumen and a seminiferous epithelium showing evidence of active spermatogenesis; the interstitial compartment was enlarged and contained numerous Leydig cells dispersed in clusters around the blood vessels (Fig. 2A).

ERα and ERβ Immunostainings

Positive ERα/ERβ immunoreactivity was detected exclusively as nuclear staining. Intensity of immunostaining was classified as negative (no staining, −), weak (+), moderate (++), and strong (+++) on the basis of microscopy observation. Similar ERα/ERβ expression patterns were detected in all the mature testes examined as well as in the immature gonads and summarized in Table 1.

ERα. In immature pig testes (Fig. 1B), no immunoreaction was detected in both germ and somatic cells. In mature testes (Fig. 2B and C), Sertoli cells were immu-

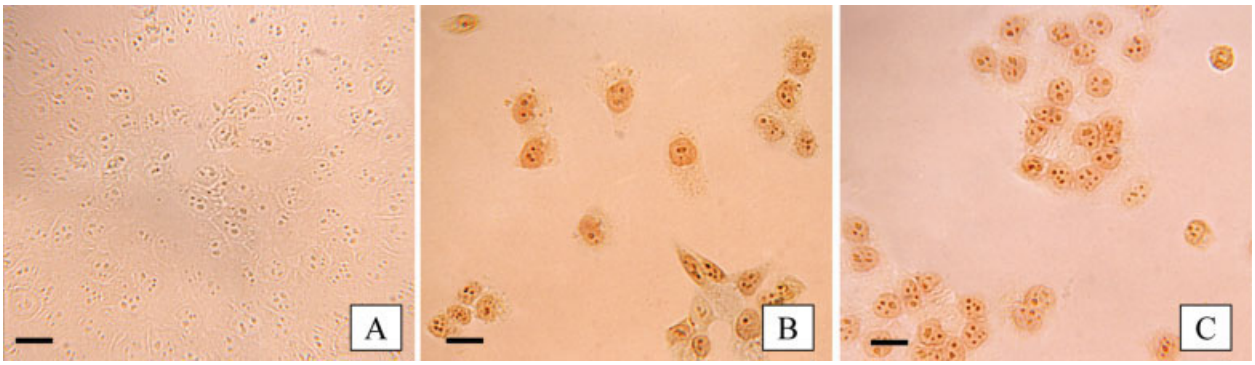

Fig. 3. Immunostainings of HeLa cells. **A:** Representative ERα/ERβ immunonegative cells transfected only with the vector. **B:** ERα immunoreactivity in HeLa cells transfected with a plasmid encoding for ERα. **C:** ERβ immunoreactivity in HeLa cells transfected with a plasmid encoding for ERβ. Scale bars = 12.5 μm.

nonegative but germ cells showed a variable immunostaining pattern. In fact, the immunoreactivity was weak in spermatogonia, strong in most spermatocytes, and absent in spermatids. In the interstitium, most Leydig cells revealed a moderate immunoreaction.

ERβ. In immature gonads (Fig. 1C and D), within the seminiferous tubules, Sertoli cells were immunonegative and occasionally weakly stained, but spermatogonia revealed strong or moderate immunostaining. In addition, peritubular myoid cells and Leydig cells showed a strong immunoreactivity. In mature gonads (Fig. 2D and E), Sertoli cells revealed no reaction but germ cells showed a differential immunoreactivity. In fact, the staining was moderate or strong in spermatogonia, strong in spermatocytes, and weak or absent in spermatids. Most peritubular myoid cells were strongly stained while Leydig cells were immunonegative.

ERα/ERβ in HeLa Cells

ERα and ERβ antibodies are available from commercial and private sources, but considerable variability in the specificity and sensitivity has been reported in different studies, particularly for ERβ (Skliris et al., 2002). The steroid receptor-negative HeLa cells have been transfected with expression plasmids encoding both ERα and ERβ to verify the specificity of antibodies. This induced a strong nuclear ERα/ERβ immunodetections (Fig. 3B and C), while the cells transfected only with the vector were immunonegative (Fig. 3A).

Western Blot Analysis

To provide further evidence that pig testes express ERα and ERβ proteins, Western blot analysis was performed on tissue extracts (Fig. 4). ERα was detected only in mature testes as a single protein band of 66 kDa, corresponding to the band detected in the extract of HeLa cells, which were transfected with a plasmid encoding ERα (Fig. 4A). ERβ protein was detected in both mature and immature testes as a single protein band of 59 kDa comigrating together with the extract of HeLa cells, transfected with a plasmid encoding ERβ (Fig. 4B). No band was present in the extracts of HeLa cells, which were transfected only with the vector (Fig. 4).

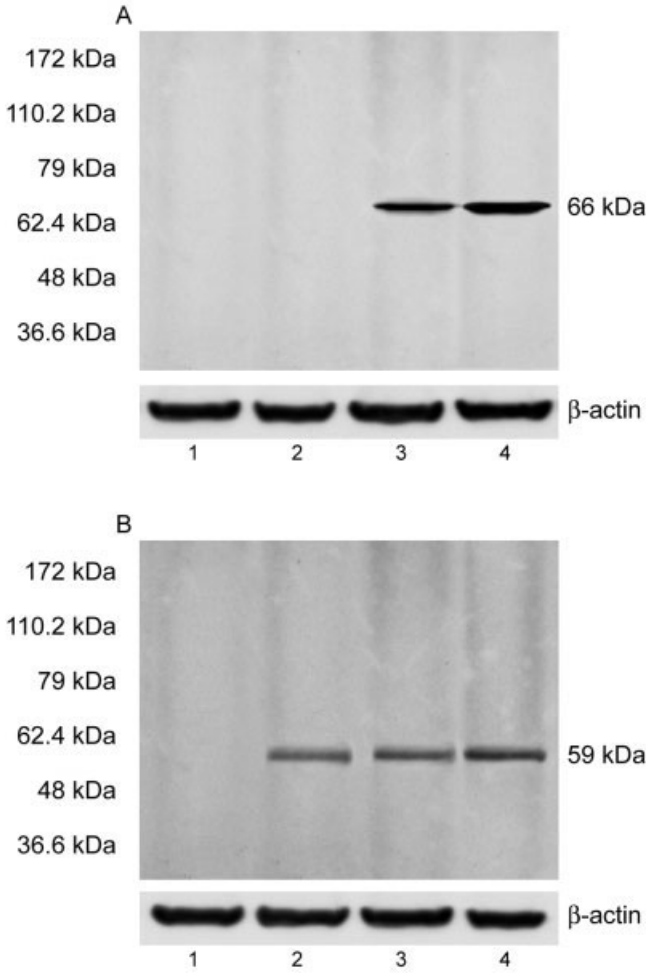

Fig. 4. Immunoblot of pig testicular protein extracts for ERα and ERβ. **A:** ERα expression. Lane 1, HeLa cells transfected only with the vector; lane 2, immature testis; lane 3, mature testis; lane 4, HeLa cells transfected with a plasmid encoding for ERα. **B:** ERβ expression. Lane 1, HeLa cells transfected only with the vector; lane 2, immature testis; lane 3, mature testis; lane 4, HeLa cells transfected with a plasmid encoding for ERβ. The number on the left corresponds to molecular mass of the marker proteins. β-actin (in A and B) serves as a loading control.

DISCUSSION

Estrogen receptors have been discovered in multiple cell types of the mammalian testes but their distribution was variable within and across the species and, sometimes, with conflicting results (Hess et al., 2001; Hess, 2003). The two subtypes, ERα and ERβ, showed distinct patterns of cellular expression (occasionally overlapping) with a prevalent distribution of ERα in Leydig cells and of ERβ in Sertoli and/or in germ cells of most mammals (van Pelt et al., 1999; Jefferson et al., 2000; Nie et al., 2002; Zhou et al., 2002).

The results of the present study have provided the first evidence on the localization of both ER subtypes in testicular cell populations of pig. Somatic and germ cells of testes showed the immunostaining exclusively in the nuclei and Western blot analysis confirmed antibody specificity.

The two receptors showed a differential expression pattern in young and adult gonads. In fact, ERα immunoreactivity was lacking in immature pig testes, as previously reported (Nielsen et al., 2001), but it was detected in Leydig cells, spermatogonia, and spermatocytes of mature gonads. Our data demonstrated that ERα distribution in prepuberal testes is different in pig compared to rat and mouse, showing ERα receptor in interstitial cells (Fisher et al., 1997; Nielsen et al., 2000), similar to the condition in fetal human testes (Takeyama et al., 2001; Gaskell et al., 2002). ERα presence in germ cells of mature male gonads distinguishes the pig from rat, mouse, cat, dog (Fisher et al., 1997; Nie et al., 2002; Zhou et al., 2002), and human (Makinen et al., 2001; Saunders et al., 2001).

Our study detected ERβ in pig testes at both the considered ages, but the pattern of immunoreactive cells was age-dependent. Prepuberal gonads showed ERβ expression in spermatogonia, peritubular myoid cells, and Leydig cells. Postpuberal testes had ERβ in spermatogonia, spermatocytes, and, outside seminiferous tubules, the immunostaining was restricted only to the peritubular myoid cells. Conversely, ERβ was detected only in seminiferous tubule cells of rat and mouse immature testes (van Pelt et al., 1999; Jefferson et al., 2000), while it was expressed in germ and interstitial cells but also in Sertoli cells of human fetal testes (Takeyama et al., 2001; Gaskell et al., 2002). Furthermore, the concomitant ERβ absence in Sertoli and Leydig cells of adult testes is a peculiarity of pigs with respect to other studied mammals and to human (Saunders et al., 1997, 2001; Jefferson et al., 2000; Makinen et al., 2001; Nie et al., 2002; Zhou et al., 2002).

Therefore, the results of the present study showed a unique distribution pattern of ERs in somatic and germ cells of young and adult pig testes, confirming the species-specific gonadal expression of estrogen receptors. However, as in other investigated species, the cellular ER distribution in pig testes appears to be developmentally regulated. This agrees to our recent data demonstrating that puberty can also influence the ERβ expression in epithelial cells of pig epididymis (Carpino et al., 2004).

The biological significance of estrogen receptors in male genital tract still needs to be clarified. Although levels of ERβ are greater than those of ERα in the male genital tract, mice lacking ERβ are fertile and have normal genital tissues (Krege et al., 1998; Dupont et al., 2000), while mice lacking ERα are infertile and exhibit remarkable morphological abnormalities of the reproductive system (Eddy et al., 1996; Hess et al., 2000). It has been proposed that ERβ could act as a negative regulatory partner for ERα (Hall and McDonnell, 1999; Weihua et al., 2000). Estrogen receptor role in human testes is less clear. In fact, the lack of ERα expression has been reported in both immature and adult gonads (Makinen et al., 2001; Gaskell et al., 2002), and normal testes and normal sperm density have been observed in an ERα-deficient man (Smith et al., 1994). Therefore, ERβ could be the receptor mediating the effect of estrogens in human testes. The recent discovery of different human ERβ isoforms with distinct cell expression patterns (Gaskell et al., 2002; Saunders et al., 2002; Scobie et al., 2002) makes the interpretation of estrogen receptor expression in male gonads more difficult.

The involvement of estrogen receptors in development and functional activity of pig testes is unknown. Recently, it has been reported that there is a functional linkage between ERβ and embryonic growth of pigs (Kowalski et al., 2002). Therefore, our study represents a basic foundation to investigate the role of ERs in porcine male gonads. In fact, the variable ERα/ERβ immunostaining pattern in testicular somatic and germ cells as well as in immature and mature gonads suggests that estrogens could modulate spermatogenesis and testis development via a differential expression of the two estrogen receptor subtypes. Further studies investigating the expression patterns of the various ERβ isoforms in testicular cells could provide greater understanding of the gonadal maturation mechanism in pig.

Currently, scientific literature discusses the possibility that environmental chemicals could induce reproductive and developmental anomalies through their effects on endocrine function (Toppari et al., 1996; Cooper and Kavlock, 1997). Particularly, environmental substances could affect the interaction between steroid receptors and their ligands; therefore, the exposure of pigs to endocrine disruptors during critical developmental periods might damage testis differentiation.

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