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Immunolocalization of Estrogen Receptor beta, Androgen Receptor and Ki-67 Protein in Testicular Tissues of Unilateral Cryptorchidism Boar

Sukanya Manee-in

Pinthira Thiengthiantham

Chanlika Prommapan

Kampon Kaeoket

Original Article

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Sukanya Manee-in* Pinthira Thiengthiantham Chanlika Prommapan Kampon Kaeoket

Abstract

This study was performed to investigate histological structure, expressions of estrogen receptor beta (ERβ), androgen receptor (AR) and proliferation marker (Ki-67 protein) in scrotal and abdominal testicular tissues of unilateral cryptorchidism prepubertal boars. Testicular tissues were obtained from 8 unilateral cryptorchidism boars. Immunohistochemical staining for ERβ, AR and Ki-67 protein was performed by avidin-biotin-peroxidase complex (ABC) method. The similar histological structure of both scrotal and abdominal testicular tissues was observed. Immunolocalization of Ki-67 was found in the nuclei of germ cells, interstitial cells and Sertoli cells of both scrotal and abdominal testicular tissues. For ERβ and AR, the immunolocalization was found in germ cells and interstitial cells of both scrotal and abdominal testicular tissues. Based on the statistical analysis, the ERβ expression in the interstitial area of scrotal testis was significantly higher than in the abdominal testis. A tendency of more expressions of Ki-67 protein (p=0.40) in the seminiferous tubule of the scrotal testicular tissues than in the abdominal testicular tissues was found. The expressions of AR in scrotal and abdominal testicular tissues were not significantly different. In conclusion, the histological structures of scrotal and abdominal testis of unilateral cryptorchidism boars are not different. However, there are differences in their immunolocalization patterns between the scrotal and abdominal testis as well as seminiferous tubules and interstitial areas.

Keywords: androgen receptor, cryptorchidism boar, estrogen receptor beta, Ki-67

Department of Clinical Science and Public Health, Faculty of Veterinary Science, Mahidol University, Salaya, Putthamonthon, Nakhon Pathom 73170, Thailand
Corresponding author E-mail: *vssukanya@mahidol.ac.th*

บทคัดย่อ

ตำแหน่งของตัวรับฮอร์โมนเอสโตรเจน ชนิดเบต้า ตัวรับฮอร์โมนแอนโดรเจน และโปรตีน Ki-67 ในเนื้อเยื่ออัณฑะของสุกรที่เป็นทองแดงข้างเดียว

สุกัญญา มณีอินทร์* ปิณฑิรา เที่ยงเธียรธรรม ฌัลลิกา พรหมมาพันธุ์ กัมพล แก้วเกษ

การศึกษาครั้งนี้มีวัตถุประสงค์ เพื่อศึกษาลักษณะทางจุลพยาธิวิทยา การแสดงออกของตัวรับฮอร์โมนเอสโตรเจนชนิดเบต้า ตัวรับฮอร์โมนแอนโดรเจน และโปรตีนงอกขยาย (โปรตีน Ki-67) ในเนื้อเยื่ออัณฑะที่อยู่ในถุงหุ้มอัณฑะ และในช่องท้องของสุกรก่อนวัยเจริญพันธุ์ที่เป็นทองแดง เนื้อเยื่ออัณฑะถูกเก็บจากสุกรอายุ 2 เดือน จำนวน 8 ตัว ใช้วิธี avidin-biotin-peroxidase complex (ABC) ของทางอิมมูนโนฮิสโตเคมีในการตรวจหาตัวรับฮอร์โมนเอสโตรเจนชนิดเบต้า ตัวรับฮอร์โมนแอนโดรเจน และโปรตีน Ki-67 ผลการศึกษา พบว่าลักษณะทางจุลพยาธิวิทยาไม่มีความแตกต่างกันระหว่างเนื้อเยื่ออัณฑะที่อยู่ในถุงหุ้มอัณฑะและภายในช่องท้อง พบการมีอยู่ของโปรตีน Ki-67 ที่เซลล์สืบพันธุ์ เซลล์ interstitial และเซลล์ Sertoli ของเนื้อเยื่ออัณฑะที่อยู่ในถุงหุ้มอัณฑะและภายในช่องท้อง และพบการมีอยู่ของตัวรับฮอร์โมนเอสโตรเจนชนิดเบต้า และตัวรับฮอร์โมนแอนโดรเจนที่เซลล์สืบพันธุ์ และเซลล์ interstitial ของอัณฑะที่อยู่ในถุงหุ้มอัณฑะและภายในช่องท้อง ผลการวิเคราะห์ทางสถิติพบการแสดงออกของตัวรับฮอร์โมนเอสโตรเจน ชนิดเบต้า ที่บริเวณ interstitial ของเนื้อเยื่ออัณฑะที่อยู่ภายในถุงหุ้มอัณฑะมากกว่าอัณฑะภายในช่องท้องอย่างมีนัยสำคัญ และพบแนวโน้มการแสดงออกของโปรตีน Ki-67 ใน seminiferous tubule ของเนื้อเยื่ออัณฑะที่อยู่ในถุงหุ้มอัณฑะมากกว่าเนื้อเยื่อที่อยู่ในช่องท้อง (p=0.40) จากการศึกษาไม่พบความแตกต่างระหว่างการแสดงออกของตัวรับฮอร์โมนแอนโดรเจนของเนื้อเยื่ออัณฑะที่อยู่ในถุงหุ้มอัณฑะและภายในช่องท้อง จากผลการศึกษาสามารถสรุปได้ว่า ลักษณะทางจุลพยาธิวิทยาของเนื้อเยื่ออัณฑะที่อยู่ภายในถุงหุ้มอัณฑะและภายในช่องท้องไม่มีความแตกต่างกันในสุกรที่เป็นทองแดง อย่างไรก็ตาม พบความแตกต่างของรูปแบบการติดสีอิมมูนโนฮิสโตเคมีระหว่างเนื้อเยื่ออัณฑะที่อยู่ภายในถุงหุ้มอัณฑะและภายในช่องท้อง และระหว่าง seminiferous tubule และบริเวณ interstitium

คำสำคัญ: ตัวรับฮอร์โมนแอนโดรเจน สุกรที่เป็นทองแดง ตัวรับฮอร์โมนเอสโตรเจน ชนิดเบต้า Ki-67

ภาควิชาเวชศาสตร์คลินิก และการสาธารณสุข คณะสัตวแพทยศาสตร์ มหาวิทยาลัยมหิดล ศาลายา พุทธมณฑล นครปฐม 73170

ผู้รับผิดชอบบทความ E-mail: *vssukanya@mahidol.ac.th*

Introduction

Cryptorchidism is a common reproductive defect, which is a failure of one testis (unilateral cryptorchidism) or both testes (bilateral cryptorchidism) to descend to the scrotum (Pinart et al., 1999[b]). The impairment of the contralateral scrotal testis may be caused by the malfunction of the cryptorchid testis (Pinart et al., 1999[a]; Pinart et al., 2002).

It is well documented that estrogen is an important hormone for male reproductive function, in which it influence spermatogenesis and Sertoli-germ cells interaction. The physiological role of estrogen in males involve several steps of sperm production and maturation. Estrogen receptors are needed to exert estrogen action. The expression of estrogen receptor beta (ERβ) is found in immature boar testicular tissue, whereas the expression of estrogen receptor alpha (ERα) is observed in testes of mature boar (Rago et al., 2004).

Androgen plays an important role in male characteristics and accessory male sex organs development. Moreover, it plays role in the initiation of spermatogenesis (Wang et al., 2009). A previous study demonstrated that the presence of androgen receptor in boar testicular tissue was related to the expression of estrogen receptors (Ramesh et al., 2007). The presence of androgen receptor (AR) in Sertoli cells and Leydig cells are associated with various processes of spermatogenesis (Wang et al., 2009).

Proliferation of several cell types in seminiferous tubules is involved in spermatogenesis. There are many cell types in testicular tissue that can be proliferated such as germ cells, Sertoli cells and Leydig cells. (Sriraman et al., 2000; Angelopoulou et al., 2008). Ki-67 is a well-established proliferation marker (Gerdes et al., 1991). A detailed in cell cycle analysis showed that the Ki-67 nuclear antigen was expressed in G1, S, G2, and mitosis, but not in resting cell in G0 phase (Gerdes et al., 1984).

Until the present, the expressions of ERβ and AR in relation to proliferation activity in testicular tissues of cryptorchidism boars have not been fully investigated. In order to understand the role of steroid receptors; ERβ and AR presented in various cell types cryptorchidism testis and normal testis in connection with the initiation of spermatogenesis, the expressions of ERβ, AR and Ki-67 protein in scrotal and abdominal testicular tissues of the same boar are investigated.

Materials and Methods

The experimental plan was approved by the Ethical Committee for Experimentation with Animals at Faculty of Veterinary Science, Mahidol University.

Animals: Eight unilateral cryptorchidism boars aged 2 months old were investigated. The animals were kept in an open house, fed with commercial diet and given water *ad libitum.*

Collection of samples: Both testes were collected from each boar by surgical excision under general anesthesia. Approximately 1x1 cm. of testicular tissue samples were fixed in 4% (w/v) paraformaldehyde for 48 hours. Thereafter they were dehydrated, embedded in paraffin and 4 μm thick sections were cut from each block and mounted on 3-aminopropyl triethoxysilane (Sigma-Aldrich, St Louis, MO) coated slides. The sections were kept until the immunohistochemical procedure was performed.

Immunohistochemistry: The specimens were deparaffinized in xylene and rehydrated in graded ethanol. After washing with distilled water, they were subjected to high-temperature antigen retrieval by incubation with 0.01M citrate buffer, pH 6.0 in a microwave at high power (800W) for 5 min followed by long heating at 240W for 3 min and at 400W for 20 min (Manee-in and Srisuwatanasagul, 2011), at 800W for 30 min (5 min x 6) for ERβ and AR. The slides were rinsed with 10mM, pH 7.4 phosphate-buffered saline (PBS). The following procedures were performed at room temperature. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min, then the tissues were rinsed with PBS. Non-specific staining was eliminated by incubating the sections with normal horse serum for 30 min. The sections were incubated with specific primary mouse monoclonal antibody to Ki-67 (clone MIB-1, dilution 1:100, Dako, Denmark) for 2 hours at room temperature, primary rabbit polyclonal antibody to ERβ (clone H-150, dilution 1:100, Santa Cruz Biotech, CA) for 3 hours at room temperature, and rabbit polyclonal antibody to AR (clone N-20, dilution 1:100, Santa Cruz Biotech, CA) for all slides were placed in a humidified chamber. After primary antibody binding, the sections were washed in PBS and incubated with the secondary antibody in a dilution of 1:200 for 30 min. After the slides were washed in PBS the sections were incubated for 30 min with horseradish peroxidase avidin biotin complex (Vectastain® ABC kit, Vector Laboratories, Inc., USA). After the final wash with PBS, the color was developed with a freshly prepared solution of 3, 3'-diaminobenzidine (DAB kit, Vector Laboratories, Inc., USA). All sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted with glycerine gelatin for investigation under a light microscope. Sections treated with non-immune serum, instead of the specific antibody, were used as negative controls. Sections from normal pig ovary, which is known to express ERβ, normal pig intestine, which is known to express Ki-67 protein, and normal testicular tissue from pubertal boar were served as positive controls.

Evaluation of immunolabelling intensity: Each tissue section was acquired with a Nikon ECLIPSE TE2000-U.The average labeling intensity percentage evaluated by image analysis (Image-Pro® PLUS 5.0 Programming software, Media Cybernetics, Inc). The percentages of positive nuclear staining (brown nuclei) were counted on 10 randomly selected fields in each compartment (seminiferous tubules and interstitial area) (Manee-in and Srisuwatanasagul, 2011). The results were presented as mean ratio of positive percentage nuclear staining.

Statistical analysis: Data were handled and statistically analyzed using the GraphPad Prism (version 4, San Diego, USA). Normal distribution of residuals from the statistical models was tested using Kolmogorov-Smirnov test. Differences in mean number of positive percentage of ERβ, AR and Ki-67 were tested using Paired-T test. Correlation between positive percentage of ERβ, AR and Ki-67 were evaluated using Pearson correlation coefficients. A *p*-value $\leq$ 0.05 was considered statistically significant.

Results

Microscopic evaluation: The similar histological structures of both scrotal and abdominal testicular tissues were observed. Both of testicular tissues were presented by small seminiferous tubules, which composed of spermatogonia and Sertoli cells. In addition, the clusters of Leydig cell were found in the interstitial area (Fig. 1). Pathological findings of both scrotal and abdominal testes were not observed.

Table 1 The percentage of Ki-67, ERβ and AR positive area (mean±SD) in the scrotal and abdominal testis of boar (n=8)

Tissues	Ki-67		ERβ		AR	
	Seminiferous tubules	Interstitial area	Seminiferous tubules	Interstitial area	Seminiferous tubules	Interstitial area
Scrotal testis	11.9±6.92	8.18±2.93	3.16±1.96	7.46±1.36[a]	4.96±1.96	3.09±2.16
Abdominal testis	9.83±2.01	7.56±1.83	3.71±3.12	2.97±1.89[b]	6.77±1.36	4.26±1.93
Overall significance	p=0.40	NS	NS	p<0.05	NS	NS

Mean (± SD) within the same column followed by the different superscript letters are significantly different ($p \leq 0.05$)

Table 2 The percentage of Ki-67, ERβ and AR positive area (mean±SD) in the seminiferous tubules and interstitial space (n=8)

Area	Ki-67		ERβ		AR	
	Scrotal testis	Abdominal testis	Scrotal testis	Abdominal testis	Scrotal testis	Abdominal testis
Seminiferous tubules	11.9±6.92	9.83±2.01 [a]	3.16±1.96	3.71±3.12	4.96±1.96	6.77±1.36 [a]
Interstitial area	8.18±2.93	7.56±1.83 [b]	7.46±1.36	2.97±1.89	3.09±2.16	4.26±1.93 [b]
Overall significance	NS	$p \leq 0.05$	NS	NS	NS	$p \leq 0.01$

Mean (±SD) within the same column followed by the different superscript letters are significantly different ($p \leq 0.05$)

Immunohistochemistry: Immunolocalization of Ki-67 was observed in the nuclei of germ cells, interstitial cells, Sertoli cells and peritubular myoid cells of both scrotal and abdominal testicular tissues. For ERβ and AR, the immunolocalization was observed in the nuclei of germ cells and interstitial cells of both scrotal and abdominal testicular tissues (Fig. 2). The percentages of ERβ, AR and Ki-67 positive cells in scrotal and abdominal testes and the different compartments of testicular tissues are shown in table 1 and 2, respectively.

In the interstitial area, a significantly higher positive percentage of ERβ was observed in the scrotal testis compared with the abdominal testis ($p<0.05$). In the scrotal testis, the percentage of ERβ positive in the interstitial cells appeared to be higher than in the seminiferous tubules ($p=0.07$).

In the abdominal testis, a significantly higher number of AR positive cells were observed in the seminiferous tubules compared with the interstitial cells. In the scrotal testis, the percentage of AR positive cells of seminiferous tubules appeared to be higher than in the interstitial area ($p=0.06$).

The percentage of Ki-67 positive cells in the seminiferous tubules was significantly higher than in the interstitial cells of abdominal testes ($p=0.04$). In the scrotal testis, the Ki-67 positive cells in the seminiferous tubules appeared to be higher than in the interstitial cells ($p \leq 0.1$). A significant difference between percentage of Ki-67 positive cells in the scrotal and abdominal testes was not found. Therefore, there was no significant correlation between the positive area of ERβ, AR and Ki-67.

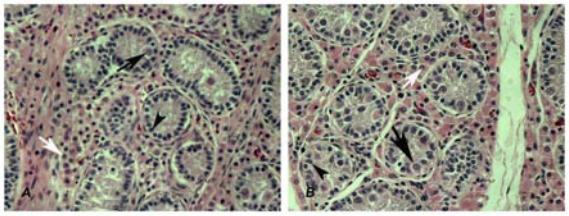

Figure 1 The morphology of the abdominal and scrotal testicular tissue of prepubertal boar testis (A and B, respectively). Sertoli cells (arrow head), spermatogonia (black arrow) and Leydig cells (white arrow). Hematoxylin-eosin staining, Bar=100 μm

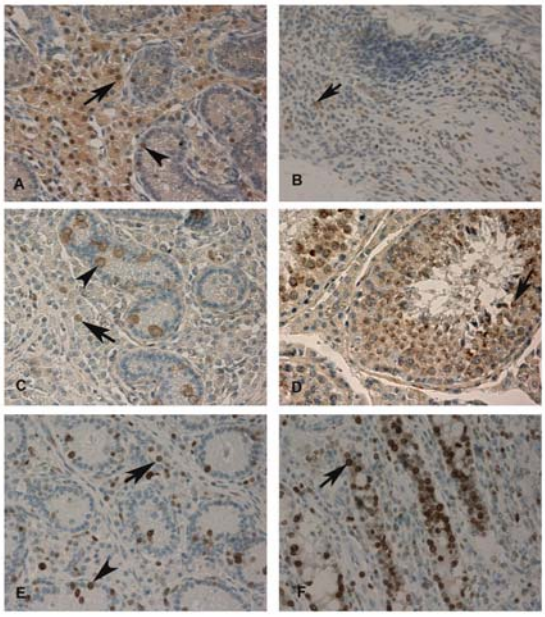

Figure 2 The expression of ERβ, AR and Ki-67 protein in prepubertal boar testicular tissues. Positive immunostaining of ERβ, AR and Ki-67 in the seminiferous tubules (arrow head) and interstitial cells (black arrow) (A, C and E, respectively). Positive control sections for ERβ, AR and Ki-67 protein (black arrow) (B, D and F, respectively), Bar=100 μm

Discussion

In the present study, similar histological findings were observed in both abdominal and scrotal testicular tissues. Both seminiferous tubules of the abdominal and scrotal testes were composed of immature Sertoli cells, a few spermatogonia and gonocyte. The Leydig cells (interstitial cells) were found in the interstitial area of the testicular tissues. These data suggested that the scrotal testicular tissues structure of prepubertal boar had not yet been fully developed (Ramesh et al., 2007).

The expression of ERβ was observed in the nuclei of germ cells and interstitial cells. This finding is similar to the previous study of the testicular tissues of immature boar (Rago et al., 2004). In this study, the ERβ expression in the interstitial area of scrotal testis was significantly higher than in the abdominal testis. This finding indicated that scrotal testis might have a good opportunity to develop, since estrogens could modulate spermatogenesis and testicular development (Rago et al., 2004). The expression of ERβ was also observed in the germ cells, which can explain the role of estrogens in germ cells development (Carani et al., 1997).

The expressions of AR in both abdominal and scrotal testes were not significantly different. This may be the result of the immaturity of the boars, in which the level of testosterone, which influence on the expression of AR, may be low (Ramesh et al., 2007). The expression of AR was pronounced in the

seminiferous tubules than another area. Similar result was found in rat testicular tissue which indicated the role of androgen in spermatogenesis (Hansson et al., 1975).

Ki-67 positive cells could be found in the seminiferous tubules and interstitial area of both the abdominal and scrotal testicular tissue. This is in accordance with previous study in cryptorchidism boar (Bernal-Manas et al., 2005). The Ki-67 positive cells were higher in the seminiferous tubules of the scrotal testis than in the abdominal testis, but there was not any significantly statistical difference. This finding may be explained by the similar histological structure of abdominal and scrotal testes found in the present study. However, when compared with an earlier study in cryptorchidism boar, the present study's result is different. The earlier study showed a lower proliferation in abdominal testicular tissues (Bernal-Manas et al., 2005). The difference may be explained by the different structures between the abdominal and scrotal testis of postpubertal boars, in which scrotal testes are fully developed. Regarding testicular tissue compartments, the Ki-67 positive cells in the seminiferous tubules were higher than in the interstitial area. This finding suggested the role of germ cell proliferation that had a higher degree than the interstitial cells.

In conclusion, this is the first report the steroid receptors protein expression in cryptorchidism testicular tissues of prepubertal boar. The expression of ERβ in the interstitial area was predominant in the scrotal testicular tissues than in the abdominal testicular tissue. A tendency of more expressions of Ki-67 receptors in the seminiferous tubule of the scrotal testicular tissues than in the abdominal testicular tissues was observed. However, the expression of AR protein in the scrotal and abdominal testicular tissues was not significant different. Therefore, a further study of steroid hormone receptors, especially AR, in cryptorchidism boar should be conducted in older boars which have fully-developed scrotal testicular structures for a better understanding of the pathogenesis and physiological status of cryptorchidism boar.

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References

Angelopoulou, R., Balla, M., Lavranos, G., Chalikias, M., Kitsos, C., Baka, S. and Kittas, C. 2008. Sertoli cell proliferation in the fetal and neonatal rat testis: A continuous phenomenon?. Acta Histochem. 110(4): 341-347.

Bernal-Manas, C.M., Morales, E., Pastor, L.M., Pinart, E., Bonet, S., Rosa Pde, L., Dolors Briz, M., Zuasti, A., Ferrer, C. and Canteras, M. 2005. Proliferation and apoptosis of spermatogonia in postpuberal boar (*Sus domesticus*) testes with spontaneous unilateral and bilateral abdominal cryptorchidism. Acta Histochem. 107(5): 365-372.

Carani, C., Qin, K., Simoni, M., Faustini-Fustini, M., Serpente, S., Boyd, J., Korach, K.S. and Simpson, E.R. 1997. Effect of testosterone and estradiol in a man with aromatase deficiency. N Engl J Med. 337(2): 91-95.

Gerdes, J., Lemke, H., Baisch, H., Wacker, H.H., Schwab, U. and Stein, H. 1984. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol. 133(4): 1710-1715.

Gerdes, J., Li, L., Schlueter, C., Duchrow, M., Wohlenberg, C., Gerlach, C., Stahmer, I., Kloth, S., Brandt, E. and Flad, H.D. 1991. Immunobiochemical and molecular biologic characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67. Am J Pathol. 138(4): 867-873.

Hansson. V., Weddington. S.C., McLean. W.S., Smith, A.A., Nayfeh, S.N., French, F.S. and Ritzen, E.M. 1975. Regulation of seminiferous tubular function by FSH and androgen. J Reprod Fertil. 44(2): 363-375.

Manee-in, S. and Srisuwatanasagul, S. 2011. Proliferation and apoptosis in normal bitch mammary tissues in relation to progesterone level. Comp Clin Path. DOI: 10.1007/s00580-010-1144-9

Pinart, E., Bonet, S., Briz, M., Pastor, L.M., Sancho, S., Garcia, N., Badia, E. and Bassols, J. 2002. Histochemical study of the interstitial tissue in scrotal and abdominal boar testes. Vet J. 163(1): 68-76.

Pinart, E., Sancho, S., Briz, M.D., Bonet, S. and Badia, E. 1999[a]. Efficiency of the process of meiosis in scrotal testes of healthy boars and unilateral abdominal cryptorchid boars. Teratology. 60(4): 209-214.

Pinart, E., Sancho, S., Briz, M.D., Bonet, S. and Garcia, N. 1999[b]. Characterization of the semen quality of postpuberal boars with spontaneous unilateral abdominal cryptorchidism on the right side. Anim Reprod Sci. 55(3-4): 269-278.

Rago, V., Maggiolini, M., Vivacqua, A., Palma, A. and Carpino, A. 2004. Differential expression of estrogen receptors (ERalpha/ERbeta) in testis of mature and immature pigs. Anat Rec A Discov Mol Cell Evol Biol. 281(2): 1234-1239.

Ramesh, R., Pearl, C.A., At-Taras, E., Roser, J.F. and Berger, T. 2007. Ontogeny of androgen and estrogen receptor expression in porcine testis: effect of reducing testicular estrogen synthesis. Anim Reprod Sci. 102(3-4): 286-299.

Sriraman, V., Rao, V.S., Sairam, M.R. and Rao, A.J. 2000. Effect of deprival of LH on Leydig cell proliferation: involvement of PCNA, cyclin D3 and IGF-1. Mol Cell Endocrinol. 162(1-2): 113-120.

Wang, R.S., Yeh, S., Tzeng, C.R. and Chang, C. 2009. Androgen receptor roles in spermatogenesis and fertility: lessons from testicular cell-specific androgen receptor knockout mice. Endocr Rev. 30(2): 119-132.