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Chronology of estrogen receptor expression in testes of mouse embryos

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Background/aim: To localize and determine the time of expression of estrogen receptors (ERs) in testes of mouse embryos by histology and immunohistochemistry.

Materials and methods: Fifty-four mature Swiss-Webster mice (*Mus musculus*) were used. Group 1 consisted of 34 mature pregnant females, 3 of which were sacrificed every day from 10.5 to 20.5 days postcoitus (dpc). One testis was removed from their embryos and processed for histology and immunohistochemistry. Group 2 consisted of 20 postnatal mice: 5 postpartum (P0) males, 5 males 4 weeks old, 5 males 8 weeks old, and 5 females 4 weeks old.

Results: The first nuclear detection of ERs was observed in embryonic male gonads at 11.5 dpc, and a highly significant increase ($P < 0.01$) was observed at embryonic days 11.5, 13.5, 15.5, 16.5, 17.5, and 19.5, with a peak at 17.5 dpc and continuing to 20.5 dpc. ER expression increased further after birth.

Conclusion: Expression of ERs occurred at certain days during mouse embryonic development, indicating the need for estrogen for certain metabolic or morphological events occurring at these days. After birth, estrogen played an important role in proliferation and maturation of certain cells in the testes. Another rise in ER expression occurred during puberty in the mature testis.

Key words: Expression of estrogen receptors, testis of mouse embryo

1. Introduction

The testes of mammals are paired organs that essentially perform two functions: production of spermatozoa and hormones, mostly androgens, and also estrogens (1). It is well known that normal testicular development and maintenance of spermatogenesis are controlled by gonadotropins and testosterone, whose effects are modulated by a complex network of factors produced locally. Among these, estrogens are concerned (2). The traditional view of estradiol as the "female" hormone and of testosterone as the "male" hormone has been challenged in recent years (3). The increased interest about the role of estrogens in the male is largely due to the demonstration that male fertility is impaired in mice lacking estrogen receptor-α (ERα) (4), or aromatase (5), along with the discovery of a second estrogen receptor-β (ERβ) (6), which is widely expressed in the male reproductive tract (7).

The androgen/estrogen balance is essential for normal sexual development and reproduction in mammals. In the mammalian testes, maintenance of this balance is under a fine-tuning via endocrine and paracrine factors, but is also related to the aromatase enzyme activity (8).

Prenatal exposure to diethylstilbestrol, a synthetic estrogen, induces abnormalities of the male genital tract of mouse and human (9).

Estrogens are synthesized in the male reproductive system in at least three different cell types of the testis: Leydig cells, Sertoli cells, and germ cells, as well as in the epithelial cells of the epididymis. In the immature testis, the primary source of estrogen is Sertoli cells (10). Currently, a growing body of evidence indicates that germ cells serve as the major source of estrogens in the male reproductive tract (11,12).

That estrogen can influence testis and epididymis function is not unexpected, given the evidence presented that estrogen biosynthesis, via the aromatase enzyme and action on its receptors (α and/or β), occurs in these tissues.

Estrogen is produced by the testes from the fetal period throughout adulthood and, similarly, ERα and β are found in the testes at all ages. While some cells express both ERα and β, such as Leydig cells, the cells in the seminiferous epithelium appear to predominantly contain ERβ.

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Evidence suggests that estrogens act at multiple levels to control, or interfere with, spermatogenesis (13).

In this study we aimed to localize and determine the time of expression of estrogen receptors (ERs) in the testes of mouse embryos by histological and immunohistochemical evaluation.

2. Materials and methods

Fifty-four mature Swiss-Webster mice (*Mus musculus*) were used. They were obtained from the animal house of the High Institute of Infertility Diagnosis And Assistant Reproductive Technology (HIID & ART)/Al-Nahrain University. The animals were used according to the general guidelines of laboratory animals (Iraqi general health law, experimental protocol section, 1981) and according to the protocol of the Laboratory Animal Center of Baghdad University, Iraq. The experimental protocol was approved by the HIID & ART/Al-Nahrain University, Baghdad, Iraq, on 2/10/2012.

Vaginal smears were performed for all the adult female mice to determine their estrus cycle stage and detect heat stage for mating (14).

Thirty-four mature pregnant females (Group 1) were sacrificed every day from 10.5 to 20.5 days postcoitus (dpc), and one testis was removed from their embryos and processed for histology and immunohistochemistry as follows: an incision was made in the abdomen to remove the live embryos from the uterus. The female embryos were discarded. Each male embryo was washed and fixed in 10% formaldehyde solution for 24 h, then stored in 70% ethanol alcohol for histology tissue processing. Sex of embryos at 10.5 and at 11.5 dpc was determined using polymerase chain reaction (PCR), by detecting the Y-chromosome (SRY gene) in the male mouse embryo.

The embryos were dissected out of their extra embryonic membranes and placed in normal saline and then fixed in 10% formaldehyde solution for 8 h to harden the tissue. The testes and pelvic region were transferred to 10% formaldehyde solution again for a further 16 h to complete fixation.

For immunohistochemical detection of ERs, 4 µm paraffin sections were mounted on Fisher brand positively charged slides (15).

Actual assessment of immunohistochemistry was performed by image analysis of tissue sections using Aperio image scope v 11.1.2.760 software and positive pixel count algorithms were used to quantify the amount of a specific color in a slide image. This algorithm has a set of default input parameters when first selected. These inputs were preconfigured for brown color quantification in the three intensity ranges (weak, positive, and strong).

Immunohistochemistry staining in testis sections of the embryonic and postnatal groups was quantified as the percentage of the positive pixels for ERs color intensity. The data were expressed as mean ± standard deviation and analyzed using Student's t-test to compare values from the embryonic and postnatal groups at individual time points. Differences between the groups were considered highly significant at $P < 0.01$, significant at $P < 0.05$, or nonsignificant at $P > 0.05$.

Group 2 consisted of 20 postnatal mice, including 5 mice postpartum zero day males (P0), 5 four-week-old males, 5 eight-week-old males, and 5 four-week-old females. One testis was removed from male mice and processed for histology and immunohistochemistry as described above. One ovary was removed from female mice and used as controls for ER detection with immunohistochemistry.

3. Results

A cell containing a nucleus with a dense brown color was considered positive for ERs (16). Sections from embryonic and postnatal mice testes showed variable intensity of immunohistochemical stain for ERs.

In the testes of mice at P0, few cells were positive for ERs. In the testis of mice at 4 weeks of age, most of the spermatogenic cells were positive (Figure 1), but the ductus epididymidis showed negative results.

Eight-week-old mice testes showed strong positive signals for anti-ER antibodies in spermatogonia, while spermatocyte and spermatid showed weak positive signals (Figure 2). Positive signals increased in intensity in the ductus epididymidis. Gonads of male embryos at 10.5, 12.5, and 14.5 dpc were negatively stained for anti-ER antibodies in primordial germ cells (PGCs), but the nuclear stain was positive in these cells at 11.5, 13.5, and 15.5 dpc. (Figure 3).

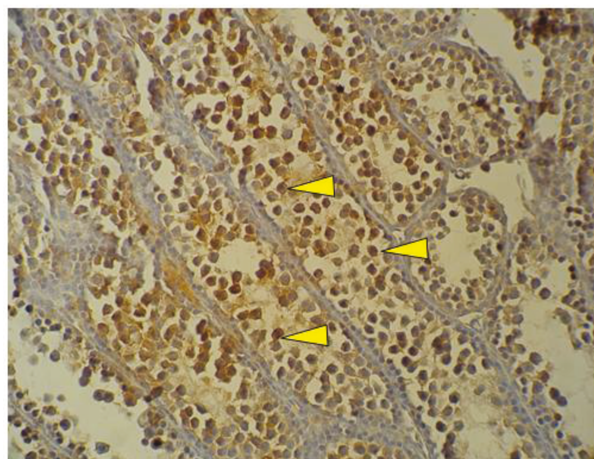

Figure 1. Four-week-old mouse testis. Immunohistochemistry for ER identification. Staining by peroxidase/DAB (brown). Positive stain in spermatogenic cells (arrow heads) (400×).

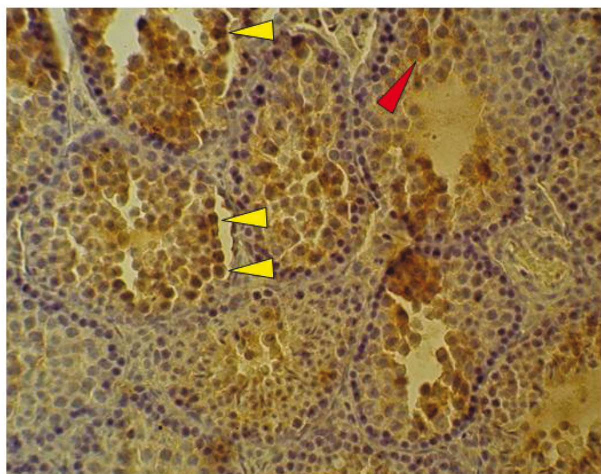

Figure 2. Eight-week-old mouse testis. Immunohistochemistry for ER identification. Positive nuclear stain in spermatogonia (yellow arrow heads) and spermatids (red arrow head). Peroxidase/DAB (brown) (400×).

Testes of embryos at 15.5 and at 16.5 dpc showed strong positive intensity for anti-ER antibodies in the peritubal cells around sex cords and weak positive intensity in PGCs inside seminiferous tubules. At 17.5 dpc there was strong positive intensity in PGCs of the seminiferous tubules and weak positive intensity in the peritubal cells around sex cords (Figure 4). Embryonic testes at 18.5 dpc showed strong positive intensity for anti-ER antibodies in both peritubal cells and in PGCs. At 19.5 dpc, positive intensity for anti-ER antibodies in PGCs inside seminiferous tubules was seen (Figure 5). Embryonic testes at 20.5 dpc showed strong positive intensity for anti-ER antibodies in PGCs inside seminiferous tubules and weak positive intensity in peritubal cells.

A 2-tailed sample statistics t-test for ER expression in the two sequenced embryonic groups showed a highly significant increase ($P < 0.01$) between embryonic days 10.5 and 11.5, 18.5 and 19.5, and between 20.5 dpc and P0. There was also a significant increase ($P < 0.05$) among embryonic days 11.5–12.5, 12.5–13.5, 15.5–16.5, 17.5–

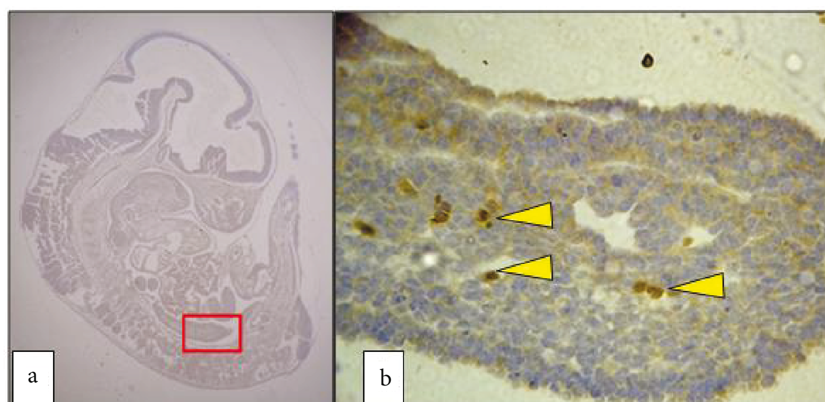

Figure 3. Section of mouse embryo at 11.5 dpc. Immunohistochemistry for ER identification. a: Whole mount sagittal section (4×). b: Gonads showing positive nuclear staining of ERs in PGCs (arrow heads). Peroxidase/DAB (brown) (400×).

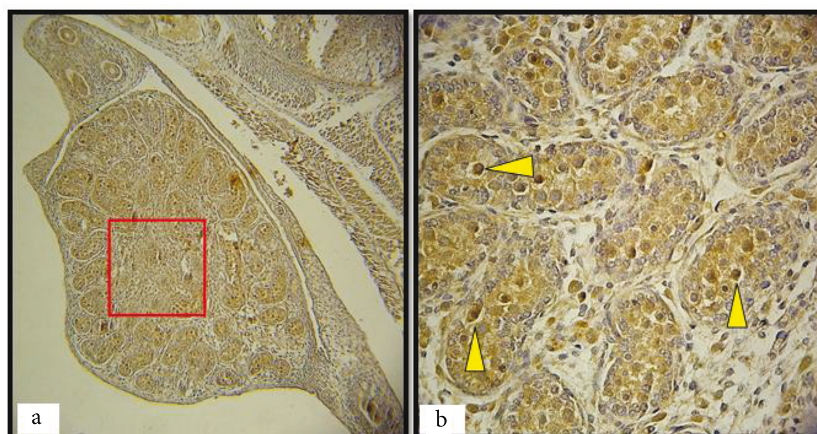

Figure 4. Section of testis of mouse embryo at 17.5 dpc. Immunohistochemistry for ERs identification. a: Sagittal section of testis (4×). b: Strong positive staining of PGCs (arrow heads). Peroxidase/DAB (brown) (400×).

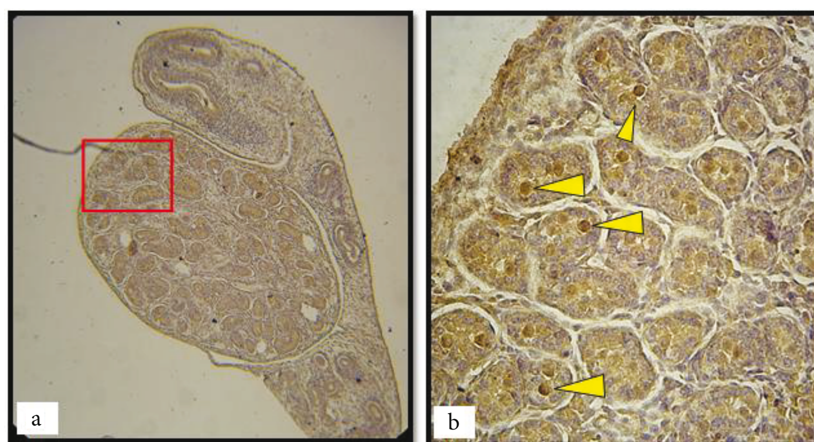

Figure 5. Section of testis of a mouse embryo at 19.5 dpc. Immunohistochemistry for ERs identification. a: Sagittal section of testis (4×). b: Strong positive staining of PGCs (arrows). Peroxidase/DAB (brown) (400×).

18.5, and 19.5–20.5, while there was a nonsignificant decrease ($P > 0.05$) among embryonic days 13.5–14.5, 14.5–15.5, and 16.5–17.5 (Tables 1 and 2; Figures 6 and 7).

The percentage of positive pixels for ER expression in embryonic male gonads showed distinct sequential elevation during embryonic life from 10.5 to 20.5 dpc (Figure 8). Comparison between percentages of positive pixels for ERs at 20.5 dpc and at P0 showed a highly significant increase at P0 (Figure 9).

4. Discussion

The sensitivity of the quantitative PCR system was high and specific for amplification of the SRY gene. It was employed on sections of all mice at the indifferent stage, 10.5 dpc, and 11.5 dpc. Positive sections were males, because the specificity was 100% for the SRY gene in male mice, and negative sections were females because the Y-chromosome was absent in female embryos. Thus, paraffin block samples of male embryos were isolated from female samples.

Table 1. Percentage of positive pixels for ER expression in gonads of male embryos.

Embryonic groups	No. of tissue sections	Percentage of positive pixels for ERs (mean ± SD)
10.5 dpc	13	7639 ± 9214
11.5 dpc	13	266,638 ± 376,548
12.5 dpc	13	112,072 ± 159,189
13.5 dpc	13	228,888 ± 304,751
14.5 dpc	13	311,711 ± 235,154
15.5 dpc	13	444,728 ± 409,341
16.5 dpc	13	831,399 ± 285,918
17.5 dpc	13	1,017,942 ± 203,415
18.5 dpc	13	745,762 ± 223,416
19.5 dpc	13	943,816 ± 142,626
20.5 dpc	13	738,419 ± 264,339

Table 2. ER expression in two sequenced embryonic groups. ** (P < 0.01) * (P < 0.05)

	ER expression mean ± SD	95% confidence interval of the difference	P-values
10.5 vs 11.5 dpc	2.589995 ± 3.79186	4.88139–2.98595	0.000**
11.5 vs 12.5 dpc	1.54566 ± 4.16832	9.73230–4.06455	0.020*
12.5 vs 13.5 dpc	1.1681± 3.67574	3.38939–1.05306	0.027*
13.5 vs 14.5 dpc	8.28224 ± 3.99677	3.24344–1.58700	0.469
14.5 vs 15.5 dpc	1.33018 ± 4.17897	3.85551–1.19515	0.273
15.5 vs 16.5 dpc	3.86670 ± 4.90208	6.82900–9.04405	0.015*
16.5 vs 17.5 dpc	1.86543 ± 3.70906	4.10680–3.75939	0.095
17.5 vs 18.5 dpc	2.72181 ± 2.80530	1.02658–4.41703	0.004*
18.5 vs 19.5 dpc	1.98055 ± 1.53050	2.90542–1.05568	0.001**
19.5 vs 20.5 dpc	2.05397 ± 2.70190	4.21233–3.68671	0.018*
20.5 vs (P0)day	1.37375 ± 2.56453	1.52873–1.21878	0.000**

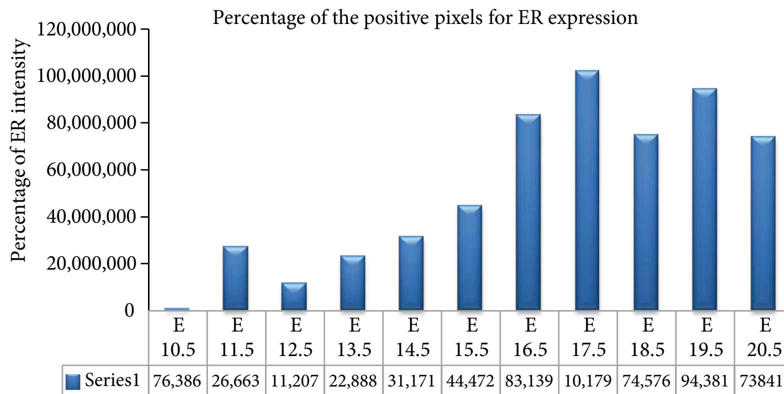

Figure 6. Percentage of positive pixels for ER expression in gonads of male embryos.

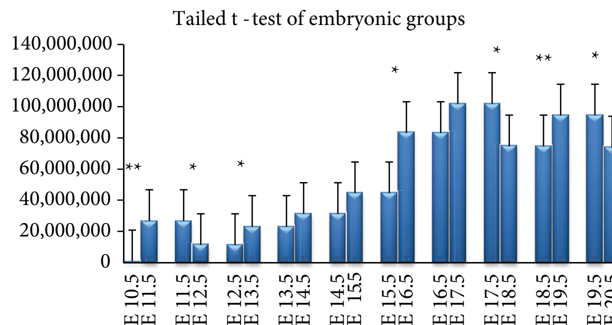

Figure 7. ER expression in two sequenced embryonic groups. ** (P < 0.01) * (P < 0.05)

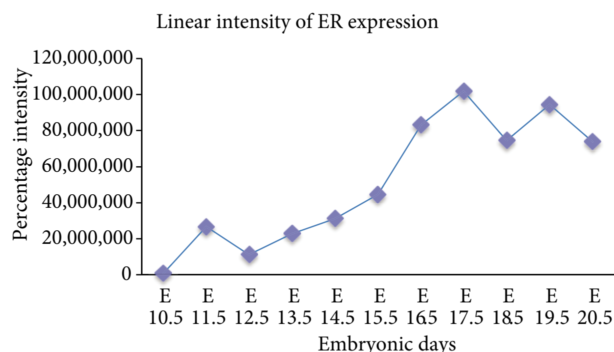

Figure 8. Quantitative evaluation of ER immunostaining.

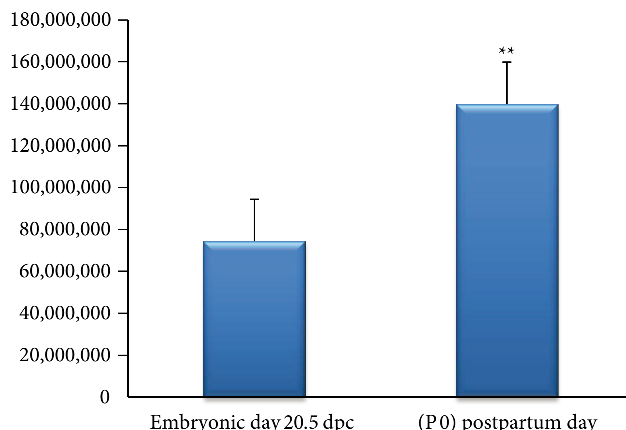

Figure 9. Percentage of positive pixels for ER expression in mice testes at 20.5 dpc and at P0.

The immunohistochemical assay in this study was used to detect the chronology of ER expression during the development of male gonads of mouse embryos from 10.5 to 20.5 dpc in addition to postnatal groups.

The results showed that there was a highly significant increase of ER expression ($P < 0.01$) at 11.5 dpc in comparison with 10.5 dpc and that the first detection of nuclear ERs at embryonic day 11.5 was restricted to a few PGCs entering the genital ridges and surrounding the sex ducts to complete their migration and reside there. Thus, estrogen was important for the process of migration of PGCs, which started at 10.5 dpc and completed at 11.5 dpc.

It was previously shown that at 11.5 dpc PGCs occupied a large area of the genital ridges to form the gonads (17,18), and it was also shown that estrogen receptors were detected in immunoblots of 10-day-old mouse embryos, with no information about the cell type (19).

At 12.5 dpc, there was no nuclear ER expression but there was a faint brown discoloration of the cytoplasm. As shown by others, this could be due to cytoplasmic synthesis or breakdown of estrogen (15).

At 12.5 dpc there was morphological overt sexual dimorphism and differentiation of gonads (20). Thus, estrogen was not needed at this stage of testis development because it did not seem to influence the process of differentiation of male gonads.

When the colonization of PGCs in genital ridges was ending at 13.5 dpc and the primordial male gonads became the prospective testes (17,18), the ERs resumed their expression. This was clearly shown by the significant increase ($P < 0.05$) of ERs at 13.5 dpc in comparison with 12.5 dpc.

At 14.5 dpc there was a negative nuclear ER expression with a faint cytoplasmic brown discoloration due to synthesis or breakdown of estrogen (15). At this stage, the prospective testis became larger than the ovary and completed differentiation (17,20).

At 15.5 dpc the nuclear ER became expressed and showed a strong positive intensity, with a significant increase ($P < 0.05$) in ERs at 16.5 dpc in comparison with 15.5 dpc. At 15.5 dpc the testes began their transabdominal descent from the abdomen to the inguinal region (21). Estrogen was shown to be important for the process of testes descent because cryptorchism developed in ER knockout mice during this stage (21,22). This explained the increase in ERs during this stage.

At 17.5 dpc there was a similar degree of positive nuclear ER expression and color intensity to 16.5 dpc. The ERs were located in peritubal cells and PGCs.

At 18.5 dpc there was a significant increase ($P < 0.05$) in ER expression compared to 17.5 dpc. This is because the transabdominal descent to the inguinal region was completed at 18.5 dpc (21).

At 19.5 dpc there was a highly significant increase ($P < 0.01$) in ER expression compared to 18.5 dpc. This was due to completing the transabdominal descent of the testis to the inguinal region (21), but with no morphological changes at 19.5 dpc (17).

When the testes matured and cells differentiated, ERs resumed their nuclear expression in the PGCs and there was a significant increase ($P < 0.05$) in ER expression at 20.5 dpc in comparison with 19.5 dpc.

In addition, there was a highly significant increase ($P < 0.01$) at P0 compared with 20.5 dpc. This was due to major morphological changes in the testes between the prenatal and the postnatal day. These changes included formation of a great number of interstitial Leydig cells and a great number of primitive Sertoli cells and large numbers of PGCs in the seminiferous tubules (22). This was in agreement with a study showing that estrogens control Leydig cells function at various stages of development (23) and other studies showing that regulation of steroidogenic genes in embryonic Leydig cells is at least partially mediated via ERα (24,25).

A highly significant increase ($P < 0.01$) in ER expression was observed in 4-week-old mice testes compared to P0. ERs were detected in cells of the seminiferous tubule, but not in interstitial Leydig cells or in the epithelial cells of ductus epididymidis. This may be because the Sertoli cells became differentiated by this point and the original large and centrally placed primordial germ cells started their meiotic division (17). In addition, some peritubular cells were transforming into smooth muscle cells and the seminiferous tubules were making lumina. At that stage, there were no spermatozoa but some tubules contained numerous young spermatids, and the ductus epididymidis contained no spermatozoa yet (17). In addition, the testes were undergoing their second stage of descent, from the inguinal region to the scrotum, and thus were in need of estrogen (26).

At 8 weeks there was a significant rise ($P < 0.05$) in ERs compared with 4-week-old mouse testes. At 8 weeks, mice testes were undergoing maturation and showed a strong positive intensity for ERs expression in spermatogonia, which were located in the basal compartment of the seminiferous tubules. Weak positive signals were found in spermatocytes and spermatids toward the lumen of seminiferous tubules, while the intensity of positive signals increased in the epithelial cells of ductus epididymidis. Our results were in agreement with others who showed the same distribution of ERs in the male reproductive tract of mice of the same age (27–29).

We can conclude that ER expression occurred at certain days during mouse embryonic development, indicating the need for estrogen for certain metabolic or morphological events occurring at that time. The first detection of ERs was at 11.5 dpc, when numerous PGCs entered the genital ridges and completed their migration. There was no ER expression at 12.5 dpc, during the overt sexual dimorphism of gonads. When colonization of PGCs in the genital ridges was ending at 13.5 dpc, there was a rise in ER expression.

ER expression was absent at 14.5 dpc, when the seminiferous tubules became solid strands of cells and the PGCs reached mitotic arrest.

Another rise in ER expression was at 15.5 dpc, during the first descent phase, from the abdomen to the inguinal region. ER expression peaked at 16.5, 17.5, and 18.5 dpc, during which the testes continued the morphological changes and completed the second descent phase from the inguinal region to the scrotum.

After birth, estrogen played an important role in proliferation and maturation of certain cells in the testes; thus other rises of ER expression were found. Another rise in ER expression occurred during puberty in the mature testis when estrogen played an important role in spermatogenesis and sperm maturation.

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