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Changes in the cellular localization of estrogen receptor alpha in the growing and regressing ovaries of *Gallus domesticus* during development

María Genoveva González-Morán *

Facultad de Ciencias, Laboratorio de Biología de la Reproducción Animal, Departamento de Biología Comparada, Universidad Nacional Autónoma de México, Mexico D.F 04510, Mexico

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ABSTRACT

In this work, the presence of estrogen receptor alpha (ER-α) was determined in different cell subpopulations in the left growing and right regressing ovaries of *Gallus domesticus* from 13-day-old chicken embryos to one-month-old chickens by immunohistochemistry. Results revealed positive ER-α immunostaining in both ovaries during development, but the percentage, staining intensity, and cellular distribution of ER-α immunostaining changes according to whether it is the left or right ovary and with the animal's age. In the left ovary, the ER-α was localized in the nuclei of the germinal epithelium and in germ cells of the ovarian cortex, as well as in the interstitial cells, undifferentiated cells, and epithelial cells of the lacunar channels of the ovarian medulla in all ages. In contrast, in the right ovary from 13-day-old chicken embryos to one-week-old chickens, only the epithelial cells of lacunar channels were ER-α immunoreactive, but in the right ovary of one-month-old chickens both the epithelial cells of lacunar channels and the interstitial cells presented ER-α. These results demonstrate differential expression of ER-α in both chicken ovaries during development in a cell type-specific distribution, suggesting that these differences may be regarded as an important cause in the process of asymmetric ovarian development in the chicken.

1. Introduction

In female chickens only the left gonad develops into a functional ovary, whereas the right gonad regresses [1]. Although development of the ovary differs between right and left sides, it is known that both, growing and regressing, ovaries of the chick embryo secrete steroid hormones and respond to gonadotropins [2,3]. In birds, estrogens are involved in the development of sexual differentiation, female secondary sexual characteristics, reproduction, and they are a fundamental component of normal ovarian function [4].

Almost all physiological effects of estrogen are mediated by specific intracellular receptors. The estrogen receptor (ER) is a member of the steroid/thyroid hormone receptor superfamily and is a ligand-depend transcription factor. It specifically binds to estrogen and regulates gene transcription via the estrogen responsive element (ERE) [5,6]. The predominant biological effects of estrogen are mediated through two distinct intracellular receptors, ER-α and ER-β, encoded by different genes in a tissue-specific manner [7,8] that have been localized in the ovaries of a wide number of species, cattle [9], sheep [10], rat [11], porcine [12] and human [13].

Information concerning the expression of ERs in the avian ovary is scarce. In chickens, the presence of two isoforms of the ER-α protein have been reported: ER-α form I (66 kDa), and ER-α form II (61 kDa). The two chicken ER-α forms differ in their ability to modulate transcription activity of estrogen target genes in a promoter-and cell type-specific manner [14]. ER-β isoform gene has been sequenced in chicken (Gen Bank # NM-204794), and its mRNA is expressed in the ovary of laying hens, showing that the expression of ER-α mRNA is higher than that of ER-β mRNA [15].

Few studies on cell-specific distribution of ER-α at the protein level in the chicken ovary have correlated both ovaries, some observations have been made only in the left ovary, mainly in the chicken embryo [16] and in laying hens [17], but not in the right one, which undergoes atrophy. Scarce information is available on both female gonads, and this is mainly focused on chicken embryos [18] and in newly hatched chicks [19], but without a sequence along development.

In a recent work from our laboratory we detected ER-α in both chicken ovaries during development by Western blot analysis [20].

* Address: Facultad de Ciencias, Laboratorio de Biología de la Reproducción Animal, Departamento de Biología Comparada, Universidad Nacional Autónoma de México, C.U., Av. Universidad 3000, México, D.F., Coyoacán, C.P. 04510, México. Fax: +52 (55) 56224828.
E-mail address: bita@live.com.mx

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However, it is not known which cellular types express ER-α in both female gonads during development.

Therefore, the purpose of the present study was to examine through immunohistochemistry the presence of ER-α in different cell subpopulations in the left growing and right regressing ovaries of *Gallus domesticus* from 13-day-old embryos to one-month-old chickens.

2. Materials and methods

2.1. Animals and histology

Fertile eggs of White leghorn chicks (Babcock-B300) were incubated at 38 °C and 58.3 ± 3% relative humidity in a forced draught incubator. Animals were studied at four different ages:13-day-old

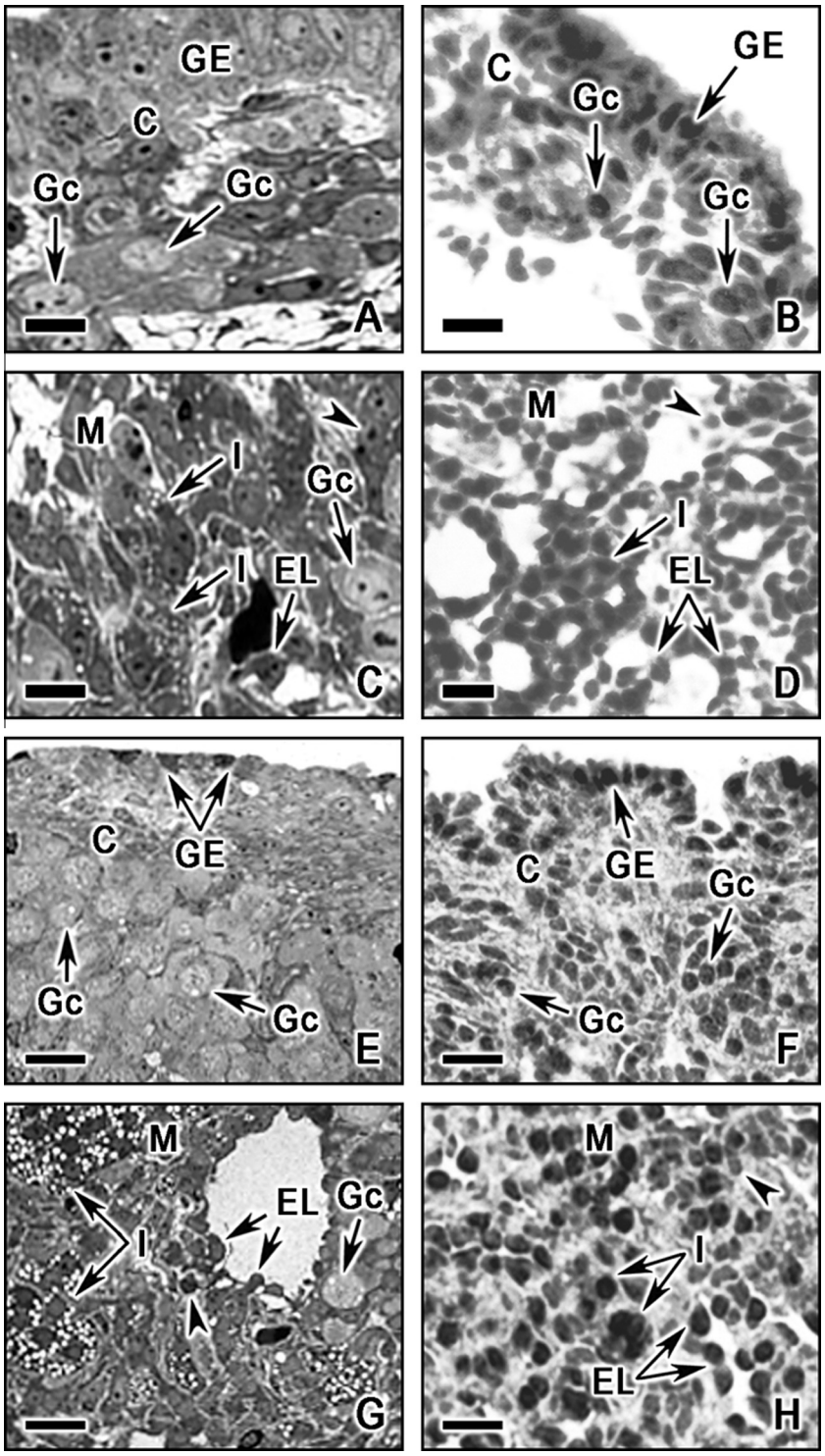

Fig. 1. Photomicrographs of the left ovary of *Gallus domesticus*. (A–D) 13-day-old chicken embryo. (A) Histology of the ovarian cortex, (B) immunolocalization of ER-α in ovarian cortex, (C) histology of the ovarian medulla, (D) immunolocalization of ER-α in ovarian medulla. (E–H) One-day-old chicken, (E) histology of the ovarian cortex, (F) immunolocalization of ER-α in ovarian cortex, (G) histology of the ovarian medulla, (H) immunolocalization of ER-α in ovarian medulla. Magnification is represented by a 10-μm scale in A–H. Cortex (C), germinal epithelium (GE), germ cells (Gc), medulla (M), interstitial cells (I), undifferentiated cells (►), and epithelial cells of lacunar channels (EL).

chicken embryo, one-day, one-week, and one-month-old chicken. Animals were housed in brooders at 30 °C under a 14:10 light–dark cycle (lights on 6:00–20:00) with food and water *ad libitum*. Eight animals per age group, were killed, the left and right ovaries were dissected immediately and cleaned from adhesive tissue, fixed in 4% paraformaldehyde for 4 h, dehydrated in graded ethanol, cleared in xylol, and embedded in paraplast. Cross sections (5 μm thick) were made and stained with hematoxylin and eosin for histological observations under light microscopy.

2.2. Immunohistochemistry

Left and right ovary sections (5 μm thick) were mounted on slides coated with poly l-lysine (Sigma, St. Louis, MO, USA). They were deparaffinized, rehydrated through graded concentrations of alcohol to distilled water. The slides were thereafter incubated in a microwave oven with 10 mM citric acid, pH 6.0, for two cycles at 750 W for 20 min each; 10 min were left between cycles. This was followed by two washes in 0.05 M sodium phosphate buffer (PBS) at pH 7.4. Then, slides were incubated in 3% hydrogen peroxide for 10 min, 0.5% triton X-100 in PBS for 20 min, 1% normal swine serum in PBS for 20 min, and with the monoclonal antibody against the ligand-binding domain of the alpha-ER (ER Ab-10; 4 μg/ml, NeoMarkers, CA) in PBS containing 0.3% triton X-100 and 0.1% gelatin for 24 h at 4 °C in a humid chamber. Sections were incubated with biotinylated secondary antibody for 30 min at room temperature, and then with streptavidin-peroxidase conjugated for 30 min. Sections were washed twice with PBS between incubations. Peroxidase activity was revealed with 3,3'diaminobenzidine chromogen solution in the presence of hydrogen peroxide. After washing, sections were dehydrated and mounted without counterstaining. Immunohistochemical negative control consisted of adjacent sections incubated with preimmune serum instead of the primary antibody.

The immunohistochemical study was performed in eight left and right ovaries; the number of ER-α immunostained cells and the staining intensity were assessed in three semi-serial sections per left and right ovaries of all ages. Immunopositive cells were considered those with brown staining of the nucleus. In the cortex of the left ovary, the total number of ER-α immunostained cells were determined in germinal epithelium cells and germ cells. In the medulla of the left ovary, the number of ER-α immunostained

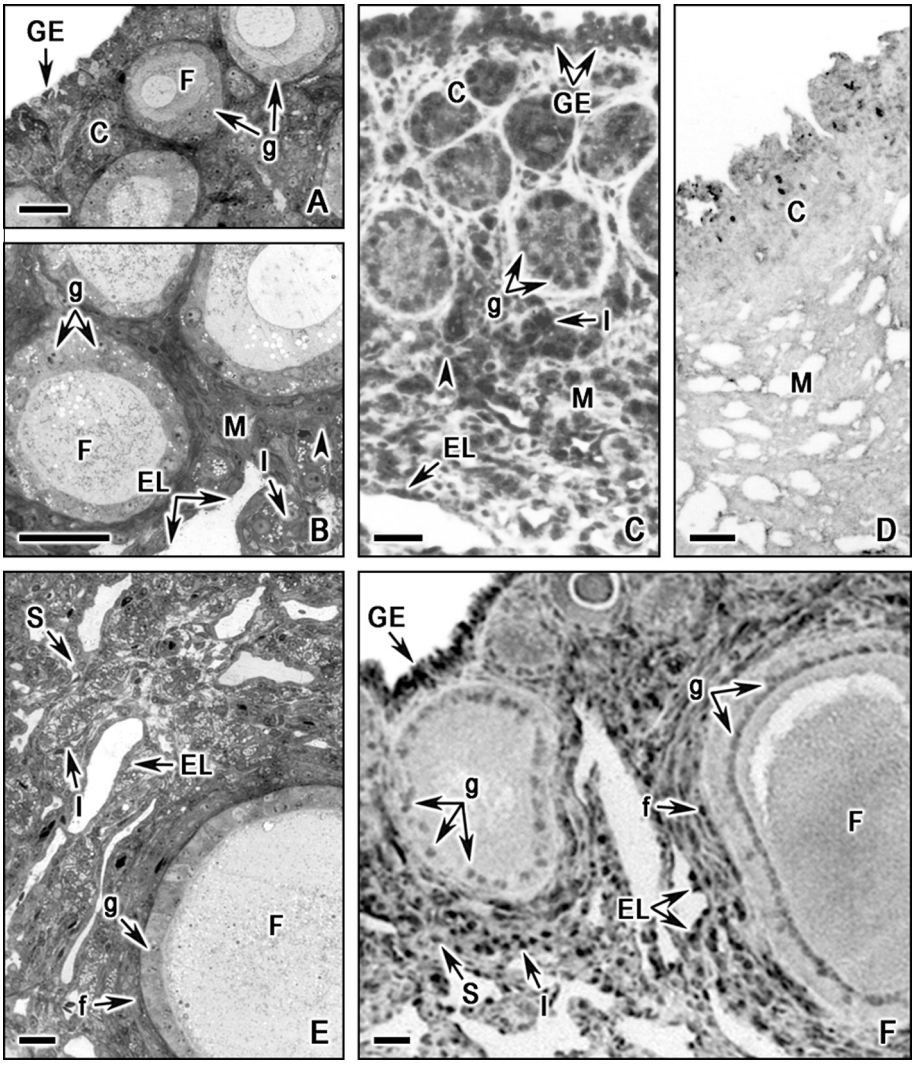

Fig. 2. Photomicrographs of the left ovary of *Gallus domesticus*. (A–C) One-week-old chicken, (A and B) histology, (C) immunolocalization of ER-α, and (D) lack of immunoreactivity in negative slides, and (E and F) one-month-old chicken, (E) histology, (F) immunolocalization of ER-α. Magnification is represented by a 10-μm scale in A–F. Cortex (C), germinal epithelium (GE), follicles (F), granulose cells (g), medulla (M), interstitial cells (I), undifferentiated cells (►), epithelial cells of lacunar channels (EL), fibroblasts (f), and stromal cells (S).

germ cells, interstitial cells, undifferentiated cells, epithelial cells of lacunar channels, granulose cells, fibroblast, and stromal cells were counted. Since the right ovary did not show a cortex, only the medulla was assessed for the total number of ER-α immunostained cells in germinal epithelium cells, germ cells, interstitial cells, undifferentiated cells, and epithelial cells of lacunar channels. The number of stained cells was expressed as percentage of immunopositive cells in relation to the total number of cells in the quantified area.

The Sigma Scan Pro program for Windows (Jandel Scientific Software, version 3) was used to analyze the staining intensity (gray values) of immunoreactive cells. The gray level was converted to a numerical value using a scale from 0 (white) to 255 (black). The background intensity was determined from sections incubated with preimmune serum instead of the primary antibody. The background was subtracted from all values. A total of 20 nuclei of each cell type per section was analyzed.

3. Results

3.1. Histological observations

Histological studies showed that the left ovary from 13-day-old chicken embryos to one-week-old chickens present cortex and medulla (Figs. 1A, C, E, G, 2A and B). The ovarian cortex presented a germinal epithelium and germ cells, but the cortex of the left ovary of one-week-old chicken presented follicles, constituted by oocytes surrounded by a single layer of granulose cells to form primordial follicles (Fig. 2A). The ovarian medulla presented germ cells, lacunar channels delimited by epithelial cells, undifferentiated cells, and interstitial cells with lipid droplets in the cytoplasm (Figs. 1C, G, and 2B). The left ovary of one-month-old chickens did not show the separation of the cortex and medulla, presenting a compact ovarian stromal tissue, with many primary follicles associated to the formation of the theca layer, which was separated

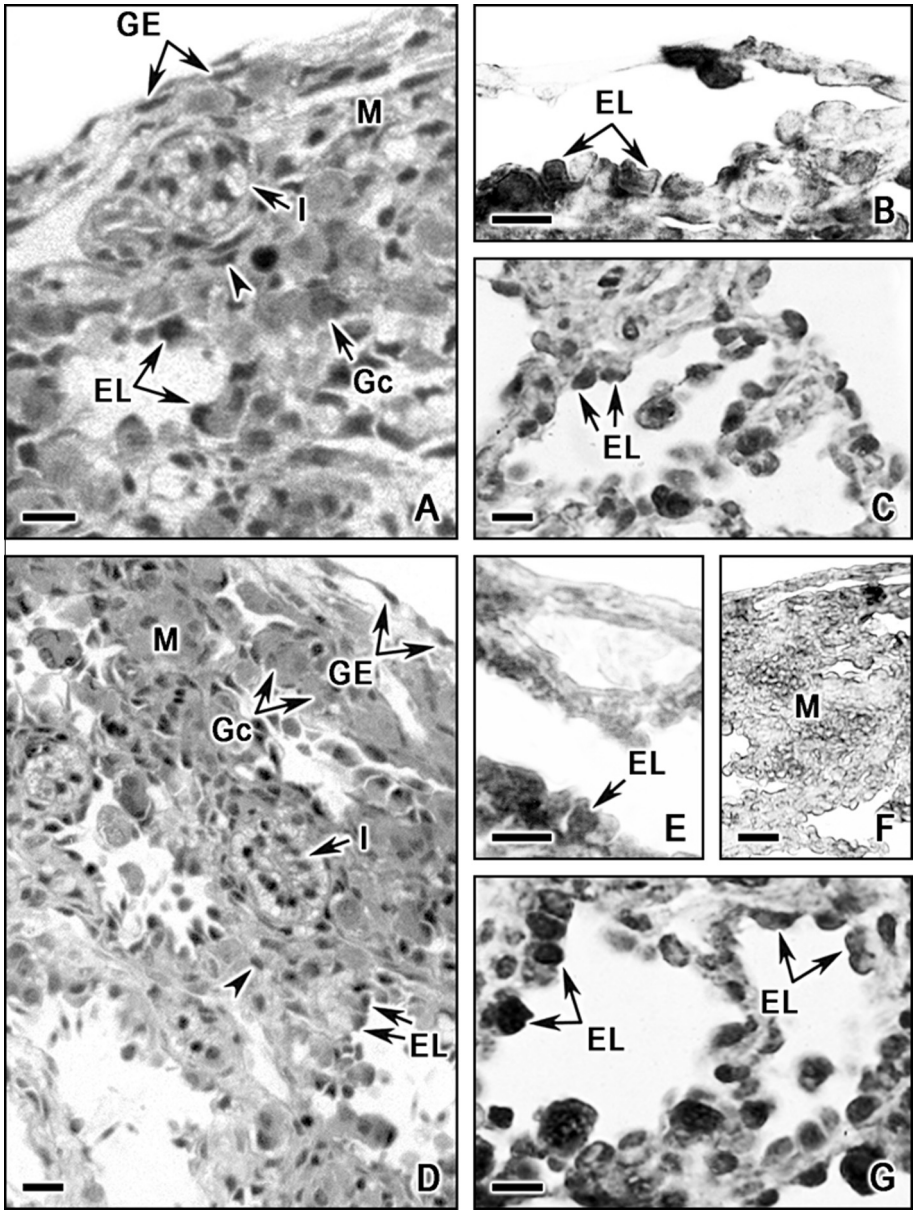

Fig. 3. Photomicrographs of the right ovary of *Gallus domesticus*. (A–C) 13-old-chicken embryo, (A) histology, (B and C) immunolocalization of ER-α. (D, E and G) One-day-old chicken, (D) histology, (E and G) immunolocalization of ER-α. (F) Lack of immunoreactivity in negative slides. Magnification is represented by a 10-μm scale in A–F and 20 μm scale G. Germinal epithelium (GE), germ cells (Gc), medulla (M), interstitial cells (I), undifferentiated cells (►), and epithelial cells of lacunar channels (EL).

from the granulose layer by the basal lamina, and remained in contact with a layer of fibroblasts (Fig. 2E).

In all examined ages, the right ovary presented a thin germinal epithelium, without a cortex, only a medulla. The medulla of the right ovary is very similar to that of the left ovary, having the same cellular components (germ cells, interstitial cells, undifferentiated cells, and epithelial cells of lacunar channels), but their organization differs according to the age of the animal (Figs. 3A, D, 4A, and D).

3.2. Localization of ER-α immunostained cells in the left chicken ovary

Immunohistochemical analysis revealed positive ER-α immunostaining in the nuclei of different cell subpopulations in both cortex and medulla of the left chick ovary in all ages studied. In the cortex of the left ovary, ER-α was predominately found in the nuclei of the germinal epithelium cells from 13-day-old chicken embryos to one-month-old chickens (Figs. 1B, F, 2C and F), in germ cells from 13-day-old embryos to one-day-old chickens (Fig. 1B and F), and in granulose cells that form primordial follicles in one-week-old chickens (Fig. 2C). The percentage and staining intensity was similar in these ER-α immunoreactive cells, except in the granulose cells in which the staining intensity was slightly weaker than in other ER-α immunoreactive cells (Table 1).

The ovarian medulla of the left ovary from 13 day-old chicken embryos to one-week-old chickens revealed ER-α immunostaining in the nuclei of the interstitial cells, undifferentiated cells, and epithelial cells of lacunar channels (Figs. 1D, H, and 2C). In all these cell types, the highest percentage and staining intensity of ER-α immunoreactive cells was found in 13-day-old chicken embryos. In one-day-old chickens, both the percentage and staining intensity of ER-α immunoreactive interstitial and undifferentiated cells decreased as compared with the previous age. In contrast, in one-week-old chickens, they were similar to those of the 13-day-old chicken embryos, although ER-α staining intensity was slightly weaker in undifferentiated cells. The percentage and staining

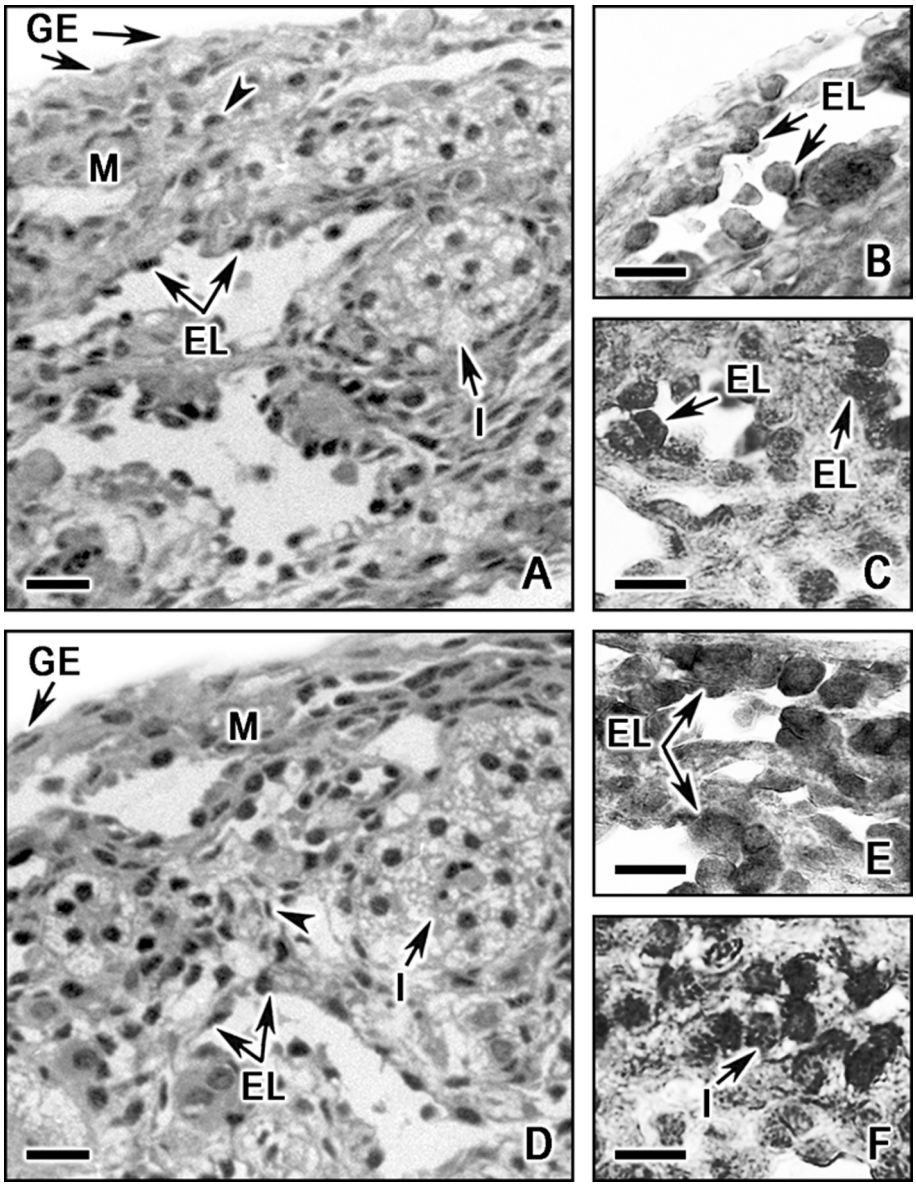

Fig. 4. Photomicrographs of the right ovary of *Gallus domesticus*. (A–C) One-week-old chicken, (A) histology, (B and C) immunolocalization of ER-α. (D–F) One-month-old chicken, (D) histology, (E and F) immunolocalization of ER-α. Magnification is represented by a 10-μm scale in A–F. Germinal epithelium (GE), germ cells (Gc), medulla (M), interstitial cells (I), undifferentiated cells (►), and epithelial cells of lacunar channels (EL).

Table 1
Percentage (P) and staining intensity (I) of ERα immunostained in different cell subpopulations of the left and right ovaries during chicken development.

Aged	Cell type	Left ovary (P)	Right ovary (P)	Left ovary (I)	Right ovary (I)
13-day-old chicken embryo	Germinal epithelium cells	96.2% ± 2.1	0%	114.7 ± 4.3	————
	Germ cells (ovarian cortex)	97.8% ± 2.3	————	113.1 ± 1.7	————
	Interstitial cells	96.4% ± 2.7	0%	116.4 ± 2.1	————
	Undifferentiated cells	95.0% ± 3.0	0%	110.6 ± 4.8	————
	Germ cells (ovarian medulla)	0%	0%		————
	Epithelial cells of lacunar channels	98.6% ± 3.4	30.1% ± 2.1	114.5 ± 1.8	88.6 ± 2.9
One-day-old chicken	Germinal epithelium cells	96.5% ± 2.6	0%	115.1 ± 3.9	————
	Germ cells (ovarian cortex)	97.2% ± 3.1	————	114.8 ± 4.2	————
	Interstitial cells	80.5% ± 3.3	0%	89.9 ± 4.0	————
	Undifferentiated cells	58.6% ± 2.1	0%	30.2 ± 2.6	————
	Germ cells (ovarian medulla)	0%	0%	————	————
	Epithelial cells of lacunar channels	91.0% ± 3.6	60.4% ± 2.6	111.6 ± 3.1	91.2 ± 3.0
One-week-old chicken	Germinal epithelium cells	98.1% ± 3.3	0%	114.8 ± 4.1	————
	Primordial follicles (granulose cells)	95.2% ± 3.0	————	92.1 ± 4.2	————
	Interstitial cells	98.6% ± 4.2	0%	114.5 ± 3.3	————
	Undifferentiated cells	92.4% ± 4.0	0%	92.3 ± 2.4	————
	Germ cells (ovarian medulla)	0%	0%	————	————
	Epithelial cells of lacunar channels	93.3% ± 3.6	80.4% ± 3.4	106.6 ± 2.8	98.6 ± 3.4
One-month-old chicken	Germinal epithelium cells	97.3% ± 3.2	0%	112.8 ± 4.4	————
	Primary follicles (granulose cells)	94.9% ± 2.9	————	50.8 ± 3.1	————
	Fibroblast	80.1% ± 3.9	————	80.2 ± 3.2	————
	Interstitial cells	94.8% ± 3.6	94.6% ± 3.9	105.6 ± 3.9	108.2 ± 2.9
	Stromal cells	20.1% ± 2.8	————	48.1 ± 2.2	————
	Epithelial cells of lacunar channels	59.8% ± 2.8	96.4% ± 28	104.6 ± 4.0	104.2 ± 3.8

Values are expressed as mean ± SD.

intensity of ER-α immunoreactive epithelial cells of lacunar channels was similar in 13-day-old chicken embryos to one-week old chickens (Table 1). Germ cells of the ovarian medulla from 13-day-old chicken embryos to one-day-old chickens were not ER-α immunoreactive (Fig. 1D and H).

The ovarian stroma tissue of one-month-old chickens presented ER-α in the nuclei of the interstitial cells, epithelial cells of lacunar channels, stroma cells, fibroblasts, and granulose cells, which gave rise to primary follicles (Fig. 2F). The percentage and staining intensity of ER-α immunoreactive interstitial cells was similar to those of the 13-day-old chicken embryos and one-week-old chickens; whereas the percentage of ER-α immunoreactive epithelial cells of lacunar channels was very low in comparison with previous ages. The staining intensity was weaker in the granulose cells of primary follicles than in the granulose cells of primordial follicles of one-week-old chickens. The percentage and staining intensity of ER-α immunostaining stromal cells was lower as compared with the other ER-α immunoreactive cells of the same age (Table 1). No staining was observed in the negative slides of the left ovary of all ages, where the primary antibody was omitted (Fig 2D).

3.3. Localization of ER-α immunostained cells in the right chicken ovary

In the right ovary from 13-day-old chicken embryos to one-week-old chickens ER-α was present only in the nuclei of the epithelial cells of lacunar channels (Figs. 3B, C, E, G, 4B and C). The percentage and staining intensity of ER-α immunoreactive epithelial cells of lacunar channels progressively increased with age (Table 1). In these aforementioned ages, the germinal epithelium, germ cells, undifferentiated cells, and interstitial cells were not ER-α immunoreactive. Interestingly, the right ovary of one-month-old chickens, presented ER-α in the nuclei of epithelial cells of lacunar channels and in interstitial cells (Fig. 4E and F). The highest percentage and strongest intensity of ER-α immunoreactive epithelial cells of lacunar channels was found in the right ovary of one-month-old chickens as compared with previous ages. The percentage and staining intensity of ER-α immunostained interstitial cells were similar to the left ovary (Table 1). No staining was observed in the negative slides of right ovaries of all ages, where the primary antibody was omitted (Fig. 3F).

4. Discussion

The histological results of the present work are in agreement with previous reports [1,21], indicating that the left ovary presents cortex and medulla, whereas the right ovary lacked cortex at all examined ages, presenting only a medulla. All these results are indicators of left ovary development, and atrophy of the right ovary, confirming the asymmetric development of both gonads. This is the first study that shows the cellular distribution of ER-α immunostaining in both ovaries during development. The immunohistochemistry analysis demonstrated that ER-α was expressed in both ovaries in all studied ages, but its percentage, staining intensity, and cellular distribution changed according to whether it is the left or right ovary, and according to the development stage of the left ovary or atrophy of the right ovary, which is related to the animaĺs age.

This study also showed that germinal epithelium cells of the left ovary highly express ER-α at all studied ages, but not in the right one. These results are consistent with previous studies performed during the early phase of gonadal development [18,22–24]. Previous evidence and the results of the present study suggest that estrogens act directly on the germinal epithelium cells through their interaction with ER-α and may regulate the development and growth of the ovarian cortex or cortical tissue in the left ovary, which increases with the age of animals [21]. The absence of ER-α in germinal epithelium cells in the right ovary may be regarded as an important cause of the unilateral ovarian development [22].

The present results and previous findings [25] showed that all germ cells (oogonia and oocytes) of the cortex of the left ovary express ER-α and PR (progesterone receptor). In contrast, medullary germ cells did no present ER-α or PR immunoreactivity, and it has been demonstrated that these germ cells never show meiosis and death via apoptosis [26,27]. All these evidences suggest that estrogens, progesterone, and adequate local microenvironment should be involved in the proliferation and development of germ cells, a process that does not occur in medullary germ cells.

In a previous work from our laboratory, we detected by Western blot analysis that ER-α expression pattern was markedly different between the left and right ovaries. ER-α was expressed in the left ovary from 13-day-old chicken embryos to one-month-old chickens, whereas in the right ovary it was only detected in one-month-old chickens [20]. In contrast, in the present work, ER-α immunostained cells were found in both ovaries in all studied ages. This discrepancy reflects the different sensitivity of Western blot and immunohistochemistry.

Although the ovarian medulla presents the same cellular components in both ovaries, the present findings indicate a cell-specific localization of ER-α in left and right ovaries related with the age of the animal. In the present study, interstitial cells, undifferentiated cells and epithelial cells of lacunar channels of the left ovarian medulla expressed ER-α in all studied ages; but, in all these cell types, the highest percentage of ER-α immunoreactive cells was localized in 13-day-old chicken embryos and in one-week-old chickens. These results could be associated with higher transcriptional effects on ER-α in these cells, because ovarian morphogenesis occurs in the13-day-old chicken embryos and it is at this age when the hypothalamic–pituitary–gonadal axis begins to function [28], and the primary oocytes become organized into primordial follicles in the one-week-old chickens [21].

In contrast, in the present work, the right ovary from 13-day-old chicken embryos to one-week-old chickens did not present ER-α immunoreactivity in interstitial and undifferentiated cells, only epithelial cells of lacunar channels showed ER-α expression. The lack of ER-α immunoreactivity in interstitial and undifferentiated cells could be related to a diminution in estrogen actions in the right ovary, which might be involved in the mechanism responsible for the regressing of the right ovary. This result also suggests that estrogen could act directly on the epithelial cells of the lacunar channels through its interaction with ER-α, which may indicate of role of estrogen in the development in this cell type, although the function of these cells is unknown. Some works have demonstrated that the lacunar zone presents voluminous spaces in functional communication with the peritoneal cavity during the embryonic stage [29]. In adult chicks, the lacunar system seems to function as an expansion room for the enlarging follicles and intervenes in follicle stalk forming [30,31], but it may have other functions, yet to be discovered.

It is interesting, that the right ovary of one-month-old chickens presents the highest percentage of ER-α immunoreactive epithelial cells of lacunar channels, and that interstitial cells also present ER-α immunoreactivity similar to that of the left ovary. This result suggests that estrogens, through their receptors, participate in the modulation of interstitial cell functions in the right ovary of one-month-old chickens. Although the right ovary regresses, some works have confirmed the steroidogenic function of interstitial cells in both ovaries [32,33], but the biological significance of the existence of ER-α in this cell type in the right ovary is still unknown, ER-α expression might be related with the hypertrophy of interstitial cells observed in the right ovary of one-month-old chickens [21], but further studies are needed.

The overall results demonstrate that ER-α is differentially expressed in both chicken ovaries during development, suggesting that both chicken ovaries have the capacity to respond to estrogens and that their effects are mediated by ER-α. These results are in agreement with previous reports [34–36] demonstrating that the differential expression of genes is involved in the process of asymmetric ovarian development in the chicken.

These differences observed in the localization of ER-α in left and right chicken ovaries, during development, suggest that there are different regulatory mechanisms for the expression of ER-α in both ovaries. The causes and relevance of these differences deserves further research.

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