

## Expression of Androgen Receptor, Estrogen Receptors Alpha and Beta and Aromatase in the Fetal, Perinatal, Prepubertal and Adult Testes of the South American Plains Vizcacha, *Lagostomus maximus* (Mammalia, Rodentia)

Candela Rocío GONZÁLEZ<sup>1</sup>), María Laura MUSCARSEL ISLA<sup>1</sup>), Noelia Paola LEOPARDO<sup>1</sup>), Miguel Alfredo WILLIS<sup>1</sup>), Verónica Berta DORFMAN<sup>1</sup>) and Alfredo Daniel VITULLO<sup>1</sup>)

<sup>1</sup>)Centro de Estudios Biomédicos, Biotecnológicos, Ambientales y Diagnóstico, CEBBAD, Universidad Maimónides, C1405BCK-Buenos Aires, Argentina

**Abstract.** Androgens and androgen receptor play a critical role in spermatogenesis and fertility in mammals, and estrogens and their receptors contribute to regulation of testicular function through initiation and maintenance of spermatogenesis and germ cell division and survival. However, results from different species are still far from establishing a clear understanding of these receptors in the different cell types from the testis. We analyzed the expression of androgen receptor, estrogen receptors  $\alpha$  and  $\beta$  and aromatase protein by immunohistochemistry and real-time PCR, in relation to proliferation followed by the expression of proliferation cell nuclear antigen (PCNA) and germinal identity by VASA protein, in fetal, perinatal, prepubertal and adult testes of *Lagostomus maximus*, a rodent with sustained germ cell proliferation and an increasing number of OCT-4-expressing gonocytes in the developing ovary. AR expression was restricted to Leydig cells and peritubular cells before sexual maturity, at which point it also became expressed in Sertoli cells. ER $\alpha$  and ER $\beta$  were expressed in seminiferous tubules and the interstitium, respectively, in both fetal and prepubertal testes. In adult testes, both ER $\alpha$  and ER $\beta$  co-localized in Leydig and peritubular cells. The aromatase enzyme, which converts androgenic precursors into estrogens, was detectable in all developmental stages analyzed and was restricted to Leydig cells. PCNA remained high until sexual maturity. ER $\alpha$  nuclear detection in germ cells and AR in Leydig cells in PCNA-positive cells suggest the possibility of a stimulatory effect of estrogens on spermatogonia proliferation. This effect might explain the increase found in VASA-expressing cells in the adult testis.

**Key words:** Androgen receptor, Aromatase, Estrogen receptors alpha and beta, *Lagostomus maximus*, Testis development  
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Androgens and the androgen receptor (AR) have been shown to play a critical role in normal spermatogenesis and fertility in mammals [1]. The testosterone, responsible for inducing meiosis, postmeiotic development and inhibiting apoptosis in the germ cell, is produced in the testis by the Leydig cells and binds to the AR modulating gene transcription in Leydig, Sertoli, peritubular and germ cells [2]. It has been clearly established that the AR is expressed in Sertoli, Leydig and peritubular cells in the mammalian testis. However, immunodetection of the AR in testicular germ cells is controversial, with reports indicating its detection and absence, although functional AR in germ cells is not essential for spermatogenesis and male fertility in mice [3].

Estrogens have been shown to largely contribute to the regulation of testicular function [4, 5], acting on the initiation and maintenance of spermatogenesis and on germinal stem cell division and survival [5]. Estrogen action is displayed by means of two different estrogen receptors (ERs), estrogen receptor-alpha (ER $\alpha$ )

and estrogen receptor-beta (ER $\beta$ ), localized in the different testicular cells types. The localization of ERs in testicular cells is not only species-specific but it also varies depending on the type of receptor and the developmental stage of the germ cell [6–10]. In most species analyzed (e.g., human, rat, cat, dog), ER $\alpha$  and ER $\beta$  co-localize in spermatogonia, spermatocytes and spermatids as well as in Sertoli, Leydig, and peritubular cells [9, 10]. In other species, such as the boar, ER $\alpha$  and ER $\beta$  localize separately to spermatogonia/primary spermatocytes and Sertoli cells, respectively [11]. In the stallion, both ERs are immunodetected in Sertoli and Leydig cells before, during and after puberty but show differential expression, with ER $\beta$  being expressed until sexual maturity is reached [11].

The synthesis of estrogens from androgenic precursors is catalyzed by the aromatase (ARO) enzyme complex situated in the endoplasmic reticulum of estrogen-producing cells [12]. It has been described that Leydig cells are the main source of testicular estrogens in mammals, since they express aromatase [6–10]. In neonatal and prepubertal animals, Sertoli cells are also a source of estrogens, and then aromatase expression diminishes from prepuberty to adulthood [13, 14].

The local effect of estrogen in the testis is not well understood. Previous studies reported inhibitory effects, such as inhibition of testosterone production under gonadotropic stimulation [15], but stimulatory actions have also been found [10]. It has been shown that

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Correspondence: AD Vitullo (e-mail: vitullo.alfredo@maimonides.edu)

estrogens induce spermatogenesis in the hypogonadal mouse [10] and proliferation of germ cells in the rat testis [16]. Nevertheless, the role of estrogens in maturation and proliferation in the mammalian testis is intricate and still far from being clearly understood.

The South American plains vizcacha, *Lagostomus maximus*, is a seasonal breeding hystricognath rodent inhabiting the Southern area of the Neotropical region. Much attention has been paid to the female germ line, since this species displays a number of unique and exceptional reproductive characteristics including massive polyovulation, abolished apoptosis-dependent germ cell attrition and follicular atresia, ovulation at mid-gestation and natural embryo selection in early post-implantation development [17–20]. However, male reproductive physiology in *L. maximus* has been mainly investigated by focusing on adult testicular changes related to the photoperiod, and endocrine variation caused by light/dark cycle fluctuation, which induces changes in the morphology of Leydig and Sertoli cells and spermatogonia [21–25]. Recently, we reported that the fetal testis of the South American plains vizcacha displays a distinctive pattern of development characterized by a sustained proliferation of germ cells with little or no apoptosis. In contrast to other mammals, a continuous rise in octamer-binding transcription factor 4 (OCT-4)-positive gonocytes, reaching 90% of germ cells, was observed in late-developing testis [26]. We report here analysis of the immunohistochemical localization of AR, ER $\alpha$ , ER $\beta$  and aromatase protein as well as their mRNA expression in the fetal, prepubertal, pubertal and adult testis of *L. maximus*. We followed age-related changes in steroid receptors in the light of proliferation of testis germ cells through immunolocalization and quantification of PCNA (proliferation cell nuclear antigen) and a germ cell-specific marker, VASA, in all developmental stages.

## Materials and Methods

### *Animals and tissue collection*

The protocol of this study was reviewed and approved by the Ethics and Research Committee of Universidad Maimónides, Argentina. Handling and euthanasia of captured animals were performed in accordance with the Canadian Council on Animal Care (CCAC) Guide for the Care and Use of Laboratory Animals (CCAA 2002) [27]. Plain vizcachas, *Lagostomus maximus*, were trapped from a resident natural population at the Estación de Cría de Animales Silvestres (ECAS), Ministry of Agriculture, Villa Elisa, Buenos Aires Province, Argentina. A total of 15 fetal testes were collected. The fetal testes were collected from fetuses at early- (n=5), mid- (n=5), and late-gestation (n=5) females classified on the basis of capture time, fetal weight and crown-heel length [28]. However, as no differences in AR, ER or ARO were detected among the gestational age, the data were grouped and are presented together in the Results as “fetal.” Perinatal (n=5), prepubertal (n=5) and adult (n=5) males were captured during the main breeding season, which extends from March to September, and were classified in groups according to body weight and testicular histology. In all cases, the animals were anesthetized with xylazine/ketamine (1:9), bled by intracardiac puncture and immediately euthanized by administration of 0.2 ml/kg body weight Euthanyl (sodium pentobarbital, sodium diphenyl hydantoinate; Brouwer, Buenos Aires, Argentina) by trained technical

staff. Fetal testes were recovered under a stereomicroscope. All samples were immediately fixed in cold 4% paraformaldehyde or kept at  $-70^{\circ}\text{C}$  for molecular analysis.

### *Immunohistochemistry*

Mounted paraffin sections (5  $\mu\text{m}$ ) were dewaxed in xylene, rehydrated in graded alcohols and washed in tap water. Endogenous peroxidase activity was inhibited in tissue sections using 0.5% v/v H<sub>2</sub>O<sub>2</sub>/methanol for 20 min at room temperature. Then, sections were blocked for 1 h with 15% normal goat serum in phosphate buffered saline (PBS) and then incubated overnight at 4 C with the primary antibody (1:200 diluted rabbit anti-AR (C-19) sc-815, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200 diluted rabbit anti-aromatase (ab18995), Abcam, Cambridge, UK; 1:200 rabbit anti-VASA, Abcam; 1:200 rabbit anti-PCNA, Abcam). After three rinses in PBS, sections were incubated for 1 h at room temperature with appropriate 1:200 diluted biotinylated secondary antibodies (Vector Labs, Peterborough, UK). After further washing in PBS, sections were incubated for 30 min with 1:100 diluted streptavidin-peroxidase complex (ABC kit, Vector Labs). Sections were then washed twice with PBS, and development of peroxidase activity was performed with 0.05% w/v 3,3'-diaminobenzidine and 0.1% v/v H<sub>2</sub>O<sub>2</sub> in Tris-HCl. Finally, sections were washed with distilled water. Negative controls were processed simultaneously by omitting the primary antibodies or preabsorbing the primary antibody with synthetic peptides.

For double immunohistochemistry, sections were first stained with rabbit anti-VASA (Abcam) using 1:100 diluted streptavidin-peroxidase complex (ABC kit, Vector Labs) for 30 min and visualized with blue VECTASTAIN (Vector Labs). After five rinses in PBS, anti-PCNA primary antibody was applied, and the subsequent steps were as described above for single immunostaining. Positively stained germ cells for PCNA and VASA were counted in single sections using an Olympus BX40 microscope (Tokyo, Japan) at 1000 $\times$  magnification. Sections were counted independently by two observers. Approximately, 500 germ cells were counted per slide. Germ cells were identified within the cords, according to their large round nuclei (larger than those from Sertoli cells) and distinctive cytoplasm.

### *Immunofluorescence*

Dewaxed and rehydrated tissue sections were blocked for 1 h with 15% normal goat serum in PBS, washed with PBS and then incubated overnight at 4 C with the primary antibody, 1:100 diluted rabbit anti-ER $\beta$  (ab3577, Abcam). After three rinses in PBS, sections were incubated for 1 h at room temperature with the appropriate 1:300 diluted anti-rabbit Alexa Fluor (Invitrogen, Carlsbad, CA, USA). After further washing in PBS, sections were incubated overnight at 4 C with the primary antibody, 1:200 diluted rabbit anti-ER $\alpha$  (MC-20, sc-542, Santa Cruz Biotechnology). After five rinses in PBS, sections were incubated in the dark for 1 h at room temperature with 1:300 diluted FITC anti-rabbit IgG (H+L) conjugate (Zymed Laboratories, San Francisco, CA, USA). Slides were mounted with DAKO fluorescence mounting medium (Dako, Carpinteria, CA, USA) and analyzed by using a Nikon C1 D-Eclipse confocal microscope (Tokyo, Japan) coupled to a Ti Eclipse fluorescence system. Negative controls were

**Table 1.** Oligonucleotide primers used for real-time PCR amplification of cDNA obtained after reverse transcription from testes of *L. maximus*

Target (accession number)	Sequence of primer (5'–3')	TM (C)	Amplified product (bp)
AR (NM_013476)	F: TGTCAAAGTGAAATGGGACC R: TGGTACTGTCCAAACGCATGT	60	74
Aromatase (NM_007810)	F: CGGGCTACGTGGATGTGTT R: GAGCTTGCCAGGCGTTAAAG	60	135
ER $\alpha$ (NM_007956)	F: CCTCCCGCCTTCTACAGGT R: CACACGGCACAGTAGCGAG	60	128
ER $\beta$ (NM_207707)	F: ACTAGTCCAAGCGCCAAGAG R: AAAGGCCTTACATCCTCACA	60	105
GAPDH (NM_008084)	F: CCAGAACATCATCCCTGCAT R: GTTCAGCTCTGGGATGACCTT	60	67

processed simultaneously by omitting the primary antibodies or preabsorbing the primary antibody with synthetic peptides.

#### RNA isolation and real time-PCR

Total testicular RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's instructions. Total RNA (3  $\mu$ g) was treated with DNaseI (Invitrogen) and used for the reverse transcription reaction in a 20  $\mu$ l reaction containing M-MLV reverse transcriptase (200 U/ $\mu$ l, Promega, Madison, WI, USA) and random hexamer primers (Biodynamics, Buenos Aires, Argentina). Reverse-transcribed cDNA was used for quantitative polymerase chain reaction (PCR) using SYBR Green PCR Master Mix and specific forward (F) and reverse (R) primers (Table 1) in a Stratagene MPX500 cyclor (Stratagene, La Jolla, CA, USA). Primers were used at a concentration of 0.3  $\mu$ M in each reaction. The cycling conditions were as follows: step 1, 10 min at 95 C; step 2, 30 sec at 95 C; step 3, 30 sec at 55 C; step 4, 30 sec at 60 C; repeating steps 2 to 4 forty-five times. Data from the reaction were collected and analyzed by the complementary computer software (MxPro3005P v4.10 Build 389, Schema 85, Stratagene, La Jolla, CA, USA). Melting curves were run to confirm the specificity of the signal. Relative quantitation of gene expression was performed using standard curves and normalized to GAPDH in each sample. For assessment of quantitative differences in the cDNA target between samples, the mathematical model of Pfaffl was applied. An expression ratio was determined for each sample by calculating  $(E_{\text{target}})^{\Delta Ct(\text{target})} / (E_{\text{GAPDH}})^{\Delta Ct(\text{GAPDH})}$ , where E is the efficiency of the primer set and Ct is the threshold cycle with  $\Delta Ct = Ct_{\text{(normalization cDNA)}} - Ct_{\text{(experimental cDNA)}}$ . The amplification efficiency of each primer set was calculated from the slope of a standard amplification curve of log (ng cDNA) per reaction vs. the Ct value ( $E = 10^{-1/\text{slope}}$ ). Efficiencies of  $2 \pm 0.1$  were considered optimal.

#### Statistical analysis

Means and standard errors (SEM) were calculated, and the GraphPad Prism Software (version 5.0 for Windows, GraphPad Software, San Diego, CA, USA) was used for one-way analysis of variance. The Newman-Keuls test was used when differences between more than two groups were compared. A P-value of less than 0.05 was considered significant.

## Results

#### Germ cell proliferation in the testis of *Lagostomus maximus*

Co-expression of the germ cell marker VASA and the proliferation marker PCNA was detected in the germ cells at all developmental stages analyzed, by double immunostaining (Fig. 1A). The percentage of PCNA-positive germ cells remained high (>80%) from the fetal to prepubertal stage, decreasing significantly (~40%) in the adult testis ( $P < 0.05$ ) (Fig. 1B). When VASA-positive germ cells were counted, a consistent increment throughout testis development was observed, and the number of VASA-positive germ cells increased significantly in prepubertal and adult testes compared with those in the fetal and perinatal stages ( $P < 0.05$ ) (Fig. 1B).

#### Testicular ontogeny of androgen receptor

AR immunostaining was detectable in the nuclei of interstitial cells, including Leydig cells and peritubular cells in fetal, perinatal, prepubertal and adult testes (Fig. 2A). In the seminiferous tubules, Sertoli and germ cells displayed a distinctive pattern of AR expression depending on the developmental stage. No AR-positive Sertoli cells and germ cells were detected in fetal and perinatal testes, whereas in prepubertal and adult testes, the majority of Sertoli cells and some germ cells were positive for AR (Fig. 2A). The expression of AR mRNA was low in the fetal and prepubertal stages, while a significant increase was observed in the perinatal group ( $P < 0.05$ ), and the highest level was observed in the adult testis ( $P < 0.05$ ) (Fig. 2B).

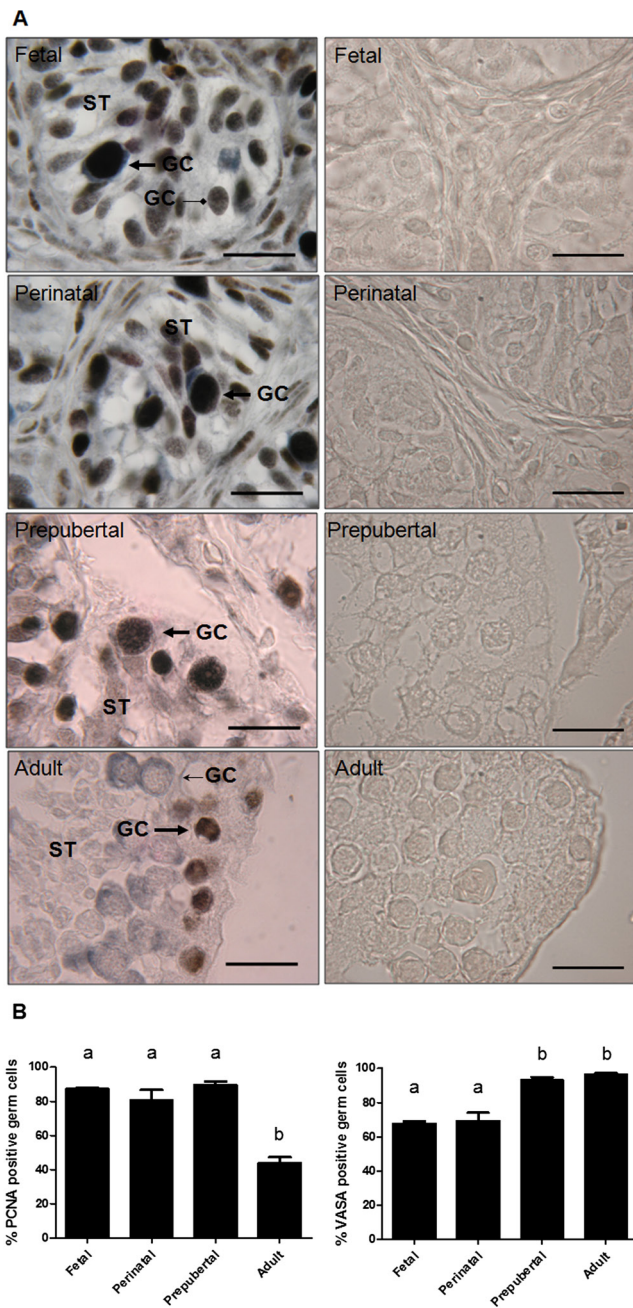
#### Immunolocalization and mRNA expression of aromatase

The expression of the aromatase enzyme was analyzed by immunohistochemistry and real time PCR in fetal, perinatal, prepubertal and adult testes (Fig. 3). In all stages studied, aromatase expression was localized in the cytoplasm of Leydig cells (Fig. 3A). Immunorexpression of aromatase was not detectable within the seminiferous tubules. Concerning the mRNA amounts of aromatase enzyme, the perinatal testis showed a significant increment in expression with respect to the other developmental stages ( $P < 0.05$ ) (Fig. 3B).

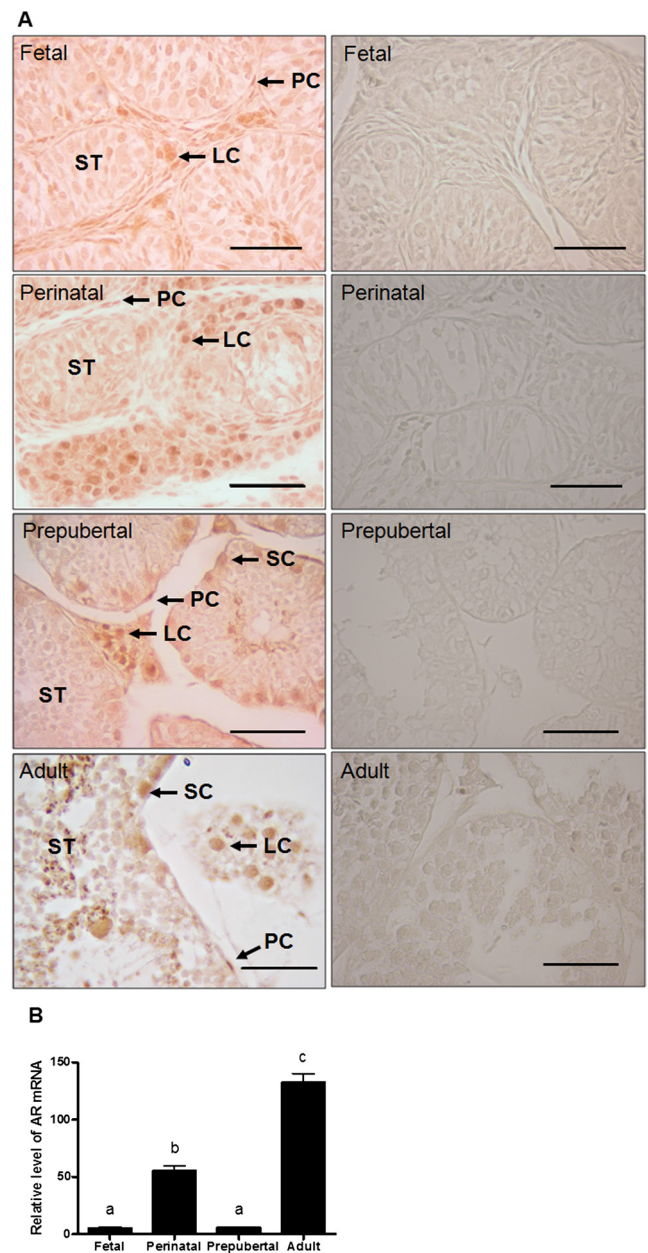
#### Testicular ontogeny of estrogen receptors $\alpha$ and $\beta$

ER $\alpha$  and ER $\beta$  were co-localized by immunofluorescence in the



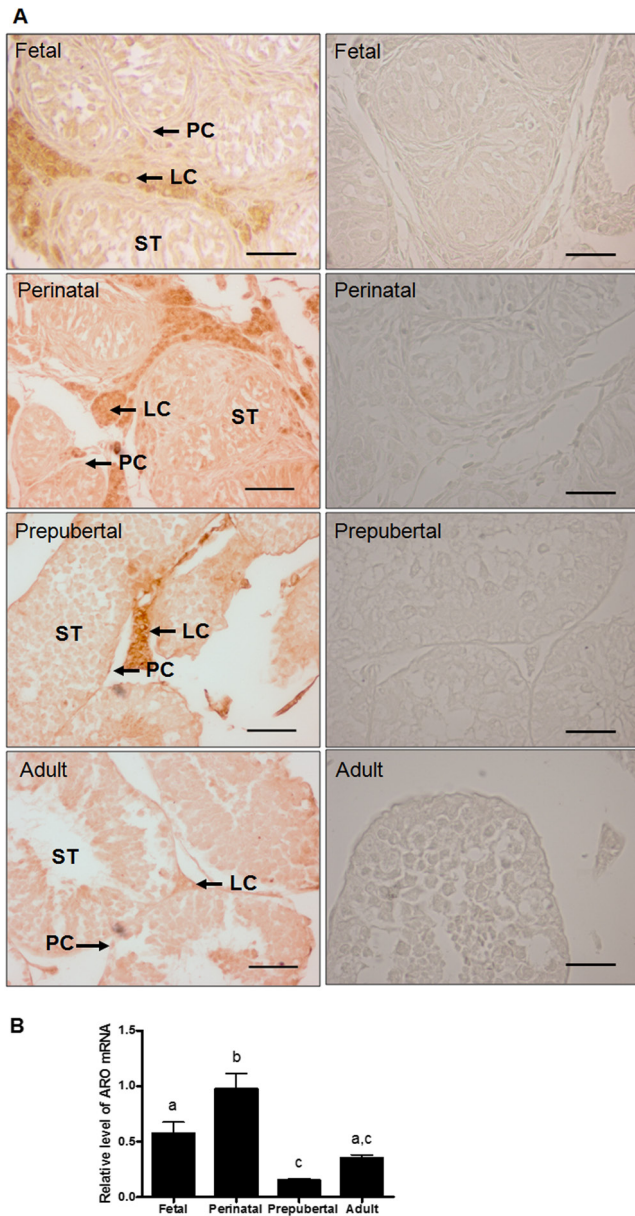


**Fig. 1.** Proliferation of germ cells throughout testis development in *L. maximus*. A: Double immunodetection of PCNA (brown nuclear staining) and VASA (blue cytoplasmic staining) proteins in fetal, perinatal, prepubertal and adult testes; the right column shows control samples treated with a synthetic peptide-preabsorbed antibody. B: Percentage of PCNA- and VASA-positive germ cells. Data are plotted as means  $\pm$  SEM. Different letters indicate significant differences between groups ( $P < 0.05$ ). Bold arrows indicate germ cells immunoreactive for VASA and PCNA, the thin arrow indicates VASA-positive/PCNA-negative germ cells, and the square-headed arrow shows a PCNA-positive/VASA-negative germ cell. GC, germ cell; LC, Leydig cell; PC, peritubular cell; SC, Sertoli cell; ST, seminiferous tubules. Scale bar: 50  $\mu$ m.



**Fig. 2.** Expression of androgen receptor (AR) throughout testis development in *L. maximus*. A: AR immunolocalization was restricted to the interstitium in all developmental stages. The right column shows representative control samples for each developmental time point, treated with a synthetic peptide-preabsorbed antibody. B: AR mRNA detection. Data are plotted as means  $\pm$  SEM. Different letters over the bars indicate significant differences between groups ( $P < 0.05$ ). LC, Leydig cell; PC, peritubular cell; ST, seminiferous tubules. Scale bar: 50  $\mu$ m.

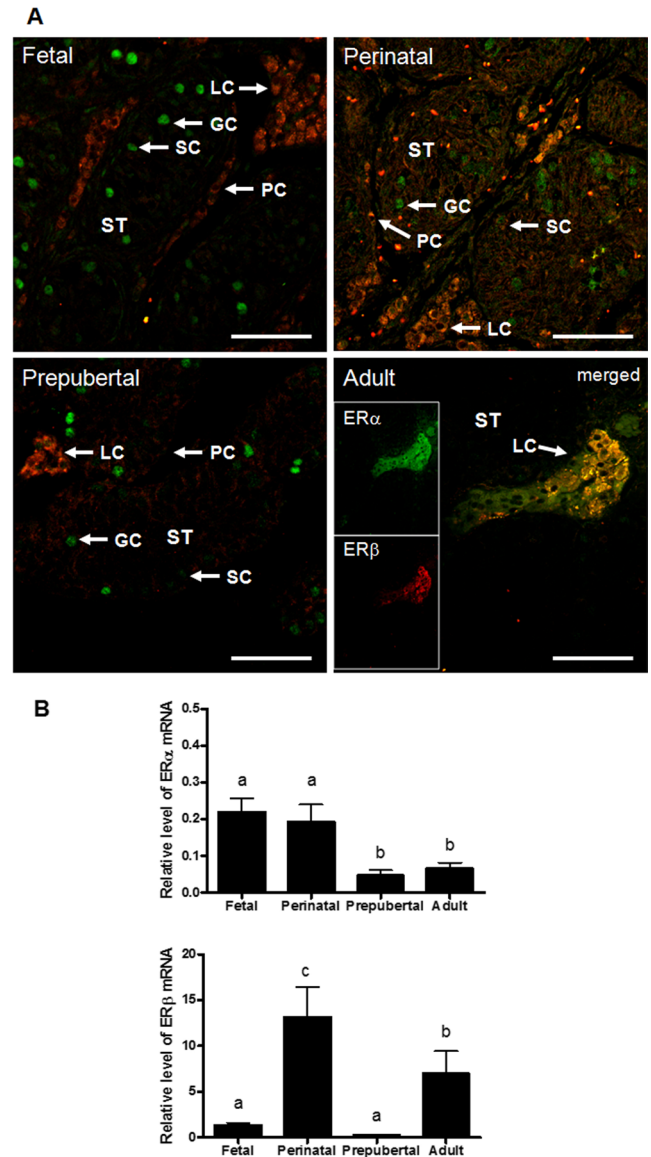
developing testis of *L. maximus*. Both ERs were detectable in the four groups studied (Fig. 4A). In fetal, perinatal, prepubertal and adult testes, ER $\beta$  was immunolocalized to the cytoplasm of interstitial cells, including Leydig cells, while no ER $\beta$ -positive Sertoli and germ cells were detected in these periods (Fig. 4A). Fetal, perinatal and



**Fig. 3.** Immunolocalization and expression of aromatase (ARO) throughout testis development in *L. maximus*. A: ARO immunostaining. The right column shows representative control sections treated with synthetic peptide-preabsorbed antibody. B: ARO mRNA levels. Data are plotted as means  $\pm$  SEM. Different letters over bars indicate significant differences between groups ( $P < 0.05$ ). LC, Leydig cell; PC, peritubular cell; ST, seminiferous tubules. Scale bar: 50  $\mu$ m.

prepubertal testes showed clear ER $\alpha$  immunostaining restricted to the nuclei of germ cells and Sertoli cells (Fig. 4A). No interstitial cells were positive for this subtype of receptor. In the adult testis, no ER $\alpha$ -positive Sertoli and germ cells were detected, and the immunostaining was restricted to the cytoplasm of Leydig cells co-localizing with ER $\beta$  (Fig. 4A).

When the expression of ERs was evaluated by real-time PCR,



**Fig. 4.** Detection of ER $\alpha$  (green staining) and ER $\beta$  (red staining) by double immunostaining throughout testis development in *L. maximus*. A: ER $\beta$  was detectable in the cytoplasm of interstitial cells, including Leydig cells, in all developmental stages. ER $\alpha$  showed nuclear localization pattern in germ cells and Sertoli cells from the fetal to prepubertal developmental periods. In the adult testis, ER $\alpha$  and ER $\beta$  co-localized in the cytoplasm of Leydig cells; note the yellow/brownish staining in the merged image. B: Relative levels of mRNA expression for ER $\alpha$  and ER $\beta$ . Data are plotted as means  $\pm$  SEM. Different letters over the bars indicate significant differences between groups ( $P < 0.05$ ). LC, Leydig cell; SC, Sertoli cell; GC, germ cell; PC, peritubular cell; ST, seminiferous tubules. Scale bar: 50  $\mu$ m.

it was observed that ER $\alpha$  mRNA was significantly diminished in prepubertal and adult testes ( $P < 0.05$ ) (Fig. 4B). On the other hand, the expression level of ER $\beta$  was very low in the fetal and prepubertal periods, increased in the adult testis and was highest in the perinatal stage (Fig. 4B).



**Table 2.** Immunohistochemical localization of AR, ARO, ER $\alpha$  and ER $\beta$  in testicular cells of *L. maximus*

Protein	Fetal				Perinatal				Prepubertal				Adult			
	LC	PC	SC	GC	LC	PC	SC	GC	LC	PC	SC	GC	LC	PC	SC	GC
AR	+	+	-	-	+	+	-	-	+	+	+	-	+	+	+	-
ARO	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-
ER $\alpha$	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-
ER $\beta$	-	-	+	+	-	-	+	+	-	-	+	+	+	+	-	-

AR, androgen receptor; ARO, aromatase; ER $\alpha$ , estrogen receptor  $\alpha$ ; ER $\beta$ , estrogen receptor  $\beta$ ; LC, Leydig cells; PC, peritubular cells; SC, Sertoli cells; GC, germ cells.

## Discussion

Our analysis of the AR and both ERs in *L. maximus* testes showed a distinctive pattern of expression in this species, which is summarized in Table 2. AR expression was restricted to the Leydig cells and peritubular cells of the interstitium until sexual maturity, at which point it was also expressed in Sertoli cells in the seminiferous tubules. Before reaching sexual maturity, ER $\alpha$  and ER $\beta$  were separately expressed in the seminiferous tubules and interstitium, respectively. In the adult testis, the expression of both ERs was restricted to the interstitium, since ER $\alpha$  stops being expressed in cells from the seminiferous tubules and are co-expressed with ER $\beta$  in Leydig and peritubular cells. Aromatase protein was detectable throughout development but was restricted to Leydig cells.

Studies in rodents have provided some insight into the maturation and differentiation of the male germ cell lineage, but little is known about the proliferation of testicular germ cells, and the balance between proliferation and cell death is still a matter of discussion [29]. We have recently reported that the fetal testis of *L. maximus* displays a distinctive pattern of development characterized by a sustained proliferation of germ cells, and in contrast to other mammals, OCT-4 positive gonocytes increased throughout development [26]. In order to analyze the pattern of expression of AR and ERs in testicular germ cells and its possible relation to the proliferation of these cells, we also followed proliferation (PCNA) and germinal identity (VASA) throughout testis development. The number of PCNA-positive germ cells remained high until the prepubertal period, while a significant decrease was observed in the adult testis. On the other hand, it was observed that the percentage of immunopositive VASA germ cells increased significantly in prepubertal and adult testes. VASA expression was comparable to previous reports indicating that mammalian VASA protein is expressed in the cytoplasm of germ cells undergoing gametogenesis until the postmeiotic stage in the testes [30]. On this basis, it is likely that a great proportion of VASA-positive germ cells become negative, since mitotic division is arrested in them in preparation for entering meiosis [9].

In agreement with previous observations in rat, mouse and human testes [32–37], AR immunostaining in *L. maximus* was observed in the nuclei of Leydig and peritubular cells, and was localized in Sertoli cells as well as in some germ cells in prepubertal and adult testes. In line with this, the expression of AR mRNA was increased significantly in adult testes, and this probably reflects the appearance of the AR in the seminiferous tubules at this stage. Identifying testicular cellular targets of androgens and estrogens seems essential,

since studies in several models have shown that steroid hormones could possibly be related to the proliferation of germ cells [16, 31].

During the testicular ontogeny in *L. maximus*, aromatase expression was only localized in the cytoplasm of Leydig cells, as reported for numerous other species [12]. No aromatase protein was localized within seminiferous tubules, indicating that Leydig cells are the principal source of estrogens in the testes of *L. maximus*. To exert a biological function, testicular estrogens have to interact with ERs, which are transcription factors that modulate the expression of specific genes [12]. ERs were detected throughout testis development in *L. maximus*, but in contrast to other rodents, ER $\beta$  was immunolocalized in the cytoplasm of Leydig cells, whereas ER $\alpha$  was detected in the seminiferous tubules and restricted to the nuclei of germ cells and Sertoli cells in fetal, perinatal and prepubertal testes. ER $\alpha$  was detectable in Leydig cells and co-localized with ER $\beta$  in adult animals only. A similar situation has been described in adult human testes in which ER $\alpha$  protein and its mRNA are found in Sertoli cells and spermatogonia [9].

Although many inhibitory actions of estrogen on Leydig, Sertoli and germ cells have been described, there is accumulating evidence that estrogen has a predominantly stimulatory effect on germ cells [37]. One of the first bits of evidence for this was a previous study showing that the administration of estrogens to neonatal rats increased the number of undifferentiated and differentiating spermatogonia [38]. In this context, Li *et al.* [16] reported that the incubation of rat gonocytes with estradiol, stimulated their proliferation. In the testis of *L. maximus*, the number of PCNA-positive germ cells remained high throughout the fetal to prepubertal periods, which is when ER $\alpha$  is expressed in the nuclei of germ cells and the AR is localized mainly in Leydig cells. This result prompted us to consider that estrogens could exert a stimulatory effect on the proliferation of spermatogonia, yielding a significant number of germ cells in the adult testes as reflected by the increased expression of VASA protein in this period. Moreover, the mRNA expression levels of aromatase enzyme were higher in the fetal and perinatal Leydig cells; thus, locally produced estrogens might act via ER $\alpha$  to stimulate spermatogonia proliferation. During the prepubertal/adult period, the disappearance of ER $\alpha$  in the seminiferous tubules could be attributed to the appearance of the AR in Sertoli cells and some germ cells enabling the spermatogenic process.

We have previously reported that there is little or no germ cell apoptosis in the fetal testis of *L. maximus* [26], and we now add that there is strong expression of ERs. Recent studies in the human testis have shown a strong immunoexpression of both ER $\alpha$  and ER $\beta$  in the

developing germ cells and that low concentrations of 17 $\beta$ -estradiol effectively inhibited male germ cell apoptosis [38]. Our results seem to associate expression of both the AR and ERs in *L. maximus* to proliferation and pinpoint a possible role for estradiol in germ cells. It has been shown that ER $\alpha$  signaling exerts an effect on germ cell survival, since it prevents apoptosis [38]. Moreover, ER $\alpha$  knock-out mice have an increased number of apoptotic germ cells, while ER $\beta$  knock-out mice have increased numbers of spermatogonial cells [39]. Lack of ER $\beta$  in the adult germ cells of *L. maximus* could lead to an increase in the number of stem cells for spermatogenesis as reported in the stallion [11]. In conclusion, the present results underline the importance of estrogens for the normal function of the developing mammalian testis. Nevertheless, further investigation including more mammalian species are needed before having a clear understanding of how estrogens regulate testicular function, since intra- and interspecies differences have been reported [9, 11]

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### References

- Chang C, Chen YT, Yeh SD, Xu Q, Wang RS, Guillou F, Lardy H, Yeh S. Infertility with defective spermatogenesis and hypotestosteronemia in male mice lacking the androgen receptor in Sertoli cells. *Proc Natl Acad Sci USA* 2004; **101**: 6876–6881. [Medline] [CrossRef]
- Welsh M, Saunders PT, Atanassova N, Sharpe RM, Smith LB. Androgen action via testicular peritubular myoid cells is essential for male fertility. *FASEB J* 2009; **23**: 4218–4230. [Medline] [CrossRef]
- Tsai MY, Yeh SD, Wang RS, Yeh S, Zhang C, Lin HY, Tzeng CR, Chang C. Differential effects of spermatogenesis and fertility in mice lacking androgen receptor in individual testis cells. *Proc Natl Acad Sci USA* 2006; **103**: 18975–18980. [Medline] [CrossRef]
- Nilsson S, Gustafsson JA. Estrogen receptor action. *Crit Rev Eukaryot Gene Expr* 2002; **12**: 237–258. [Medline] [CrossRef]
- Hess RA. Estrogen in the adult male reproductive tract: a review. *Reprod Biol Endocrinol* 2003; **1**: 52. [Medline]
- Hess RA, Gist DH, Bunick D, Lubahn DB, Farrell A, Bahr J, Cooke PS, Greene GL. Estrogen receptor ( $\alpha$  &  $\beta$ ) expression in the recurrent ducts of the adult male rat reproductive tract. *J Androl* 1997; **18**: 602–611. [Medline]
- Sharpe RM. The roles of oestrogen in the male. *Trends Endocrinol Metab* 1998; **9**: 371–377. [Medline] [CrossRef]
- Couse JF, Korach KS. Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr Rev* 1999; **20**: 358–417. [Medline] [CrossRef]
- O'Donnell L, Robertson KM, Jones ME, Simpson ER. Estrogen and spermatogenesis. *Endocr Rev* 2001; **22**: 289–318. [Medline] [CrossRef]
- Hess RA, Bunick D, Bahr J. Oestrogen, its receptors and function in the male reproductive tract—a review. *Mol Cell Endocrinol* 2001; **178**: 29–38. [Medline] [CrossRef]
- Pearl CA, Mason H, Roser JF. Immunolocalization of estrogen receptor alpha, estrogen receptor beta and androgen receptor in the pre-, peri- and post-pubertal stallion testis. *Anim Reprod Sci* 2011; **125**: 103–111. [Medline] [CrossRef]
- Carreau S, Lambard S, Delalande C, Denis-Galeraud I, Bilinska B, Bourguiba S. Aromatase expression and role of estrogens in male gonad: a review. *Reprod Biol Endocrinol* 2003; **1**: 35. [Medline]
- Payne AH, Youngblood GL. Regulation of expression of steroidogenic enzymes in Leydig cells. *Biol Reprod* 1995; **52**: 217–225. [Medline] [CrossRef]
- Sharpe RM. The 'oestrogen hypothesis'—where do we stand now? *Int J Androl* 2003; **26**: 2–15. [Medline] [CrossRef]
- Ramaswamy S. Pubertal augmentation in juvenile rhesus monkey testosterone production by invariant gonadotropic stimulation is inhibited by estrogen. *J Clin Endocrinol Metab* 2005; **90**: 5866–5875. [Medline] [CrossRef]
- Li H, Papadopoulos V, Vidic B, Dym M, Culty M. Regulation of rat testis gonocyte proliferation by platelet-derived growth factor and estradiol: identification of signaling mechanisms involved. *Endocrinology* 1997; **138**: 1289–1298. [Medline] [CrossRef]
- Weir BJ. The management and breeding of some more hystricomorph rodents. *Lab Anim* 1970; **4**: 83–97. [Medline] [CrossRef]
- Weir BJ. The reproductive physiology of the plains vizcacha, *Lagostomus maximus*. *J Reprod Fertil* 1971; **25**: 355–363. [Medline] [CrossRef]
- Weir BJ. The reproductive organs of the female plains vizcacha, *Lagostomus maximus*. *J Reprod Fertil* 1971; **25**: 365–373. [Medline] [CrossRef]
- Jensen F, Willis MA, Albamonte MS, Espinosa MB, Vitullo AD. Naturally suppressed apoptosis prevents follicular atresia and oocyte reserve decline in the adult ovary of *Lagostomus maximus* (Rodentia, Caviomorpha). *Reproduction* 2006; **132**: 301–308. [Medline] [CrossRef]
- Calvo JC, Sagripanti JL, Peltzer LE, Guzman JA, Charreau EH. Photoperiod follicle-stimulating hormone receptors and testicular function in viscacha (*Lagostomus maximus maximus*). *Biol Reprod* 1986; **35**: 822–827. [Medline] [CrossRef]
- Hikim AP, Amador AG, Klemcke H, Bartke A, Russel LD. Correlative morphology and endocrinology of Sertoli cells in hamsters testis in active and inactive states of spermatogenesis. *Endocrinology* 1989; **125**: 1829–1843. [Medline] [CrossRef]
- Hikim AP, Amador AG, Bartke A, Russel LD. Structure/function relationship in active and inactive hamster Leydig cells: a correlative morphometric and endocrine study. *Endocrinology* 1989; **125**: 1844–1856. [Medline] [CrossRef]
- Fuentes LB, Caravaca N, Pelzer LE, Scardapane LA, Piezzi RS, Guzman JA. Seasonal variations in the testis and epididymis of viscacha (*Lagostomus maximus maximus*). *Biol Reprod* 1991; **45**: 493–497. [Medline] [CrossRef]
- Muñoz EM, Fogal T, Domínguez S, Scardapane L, Piezzi RS. Ultrastructural and morphometric study of the Sertoli cell of the viscacha (*Lagostomus maximus maximus*) during the annual reproductive cycle. *Anat Rec* 2001; **262**: 176–185. [Medline] [CrossRef]
- Gonzalez CR, Muscársel Isla ML, Fraunhoffer NA, Leopardo NP, Vitullo AD. Germ cell differentiation and proliferation in the developing testis of the South American plains viscacha, *Lagostomus maximus* (Mammalia, Rodentia). *Zygote* 2012; **20**: 219–227. [Medline] [CrossRef]
- CCAC. Guía para el cuidado y uso de animales de laboratorio. Mexican edition. Mexico City: National Academy Press; 2002.
- Leopardo NP, Jensen F, Willis MA, Espinosa MB, Vitullo AD. The developing ovary of the South American plains viscacha, *Lagostomus maximus* (Rodentia, Mammalia): massive proliferation with no sign of apoptosis-mediated germ cell attrition. *Reproduction* 2011; **141**: 633–641. [Medline] [CrossRef]
- Wilhelm D, Palmer S, Koopman P. Sex determination and gonadal development in mammals. *Physiol Rev* 2007; **87**: 1–28. [Medline] [CrossRef]
- Noce T, Okamoto-Ito S, Tsunekawa N. Vasa homolog genes in mammalian germ cell development. *Cell Struct Funct* 2001; **26**: 131–136. [Medline] [CrossRef]
- Mi Y, Zhang C, Xie M, Zeng W. Effects of follicle-stimulating hormone and androgen on proliferation of cultured testicular germ cells of embryonic chickens. *Gen Comp Endocrinol* 2004; **138**: 237–246. [Medline] [CrossRef]
- Vornberger W, Prins G, Musto NA, Suarez-Quian CA. Androgen receptor distribution in rat testis: new implications for androgen regulation of spermatogenesis. *Endocrinology* 1994; **134**: 2307–2316. [Medline] [CrossRef]
- Bremner WJ, Millar MR, Sharpe RM, Saunders PT. Immunohistochemical localization of androgen receptors in the rat testis: evidence for stage-dependent expression and regulation by androgens. *Endocrinology* 1994; **135**: 1227–1234. [Medline] [CrossRef]
- Suárez-Quian CA, Martínez-García F, Nistal M, Regadera J. Androgen receptor distribution in adult human testis. *J Clin Endocrinol Metab* 1999; **84**: 350–358. [Medline] [CrossRef]
- Zhu LJ, Hardy MP, Inigo IV, Huhtaniemi I, Bardin CW, Moo-Young AJ. Effects of androgen on androgen receptor expression in rat testicular and epididymal cells: a quantitative immunohistochemical study. *Biol Reprod* 2000; **63**: 368–376. [Medline] [CrossRef]
- Zhou Q, Nie R, Prins GS, Saunders PT, Katzenellenbogen BS, Hess RA. Localization of androgen and estrogen receptors in adult male mouse reproductive tract. *J Androl* 2002; **23**: 870–881. [Medline]
- Kula K. Induction of precocious maturation of spermatogenesis in infant rats by human menopausal gonadotropin and inhibition by simultaneous administration of gonadotropins and testosterone. *Endocrinology* 1988; **122**: 34–39. [Medline] [CrossRef]
- Pentikäinen V, Erkkilä K, Suomalainen L, Parvinen M, Dunkel L. Estradiol acts as a germ cell survival factor in the human testis *in vitro*. *J Clin Endocrinol Metab* 2000; **85**: 2057–2067. [Medline] [CrossRef]
- Gould ML, Hurst PR, Nicholson HD. The effect of oestrogen receptors alpha and beta on testicular cell number and steroidogenesis in mice. *Reproduction* 2007; **134**: 271–279. [Medline] [CrossRef]