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Expression of Aromatase, Estrogen Receptor α and β, Androgen Receptor, and Cytochrome P-450$_{scc}$ in the Human Early Prepubertal Testis

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ABSTRACT: The expression of aromatase, estrogen receptor α (ERα) and β (ERβ), androgen receptor (AR), and cytochrome P-450 side chain cleavage enzyme (cP450$_{scc}$) was studied in prepubertal testis. Samples were divided in three age groups (GRs): GR1, newborns (1- to 21-d-old neonates, $n = 5$); GR2, postnatal activation stage (1- to 7-mo-old infants, $n = 6$); GR3, childhood (12- to 60-mo-old boys, $n = 4$). Absent or very poor detection of ERα by immunohistochemistry in all cells and by mRNA expression was observed. Leydig cells (LCs) of GR1 and GR2 showed strong immunostaining of aromatase and cP450$_{scc}$ but weak staining of ERβ and AR. Interstitial cells (ICs) and Sertoli cells (SCs) expressed ERβ, particularly in GR1 and GR2. Strong expression of AR was found in peritubular cells (PCs). For all markers, expression in GR3 was the weakest. In germ cells (GCs), *i.e.* gonocytes and spermatogonia, aromatase and ERβ were immunoexpressed strongly whereas no expression of ERα, AR, or cP450$_{scc}$ was detected. It is proposed that in newborn and infantile testis, testosterone acting on PCs might modulate infant LC differentiation, whereas the absence of AR in SCs prevents development of spermatogenesis. The role of estrogen is less clear, but it could modulate the preservation of an adequate pool of precursor LCs and GCs. **(*Pediatr Res* 60: 740–744, 2006)**

During prepuberty, human and primate testes undergo profound morphologic (1) and functional changes (2,3). Indeed, in childhood, and particularly in newborns and infants, there is an active process of cell growth (4), differentiation, and transient functional activity that might program future adult function. It is remarkable that the relatively high levels of testosterone (5), inhibin B (6), luteinizing hormone, and follicle-stimulating hormone (7) described in infant boys, are not associated with concomitant maturation changes in the seminiferous cords, *i.e.* maturation of SCs and development of spermatogenesis. On the other hand, it has been proposed that the maturational events taken place in the testis during infancy might affect adult testicular cell mass, as well as testicular function (8).

In humans (9), there are three growth phases of LCs during testicular development. Fetal LCs produce testosterone required for fetal masculinization and Insl-3, necessary for testicular descent (10). They regress during the third trimester of pregnancy. A second wave of infantile LCs has been described during the postnatal surge of luteinizing hormone/testosterone in the first trimester of postnatal life (11). Finally, the last wave of adult LCs coincides with pubertal development.

The role played by androgens and estrogens in the development and function of LC is not clear. The effect of estrogen might be complex because the information generated by studies in rodents is contradictory. Some evidence suggests that, in monkeys, LC number is inhibited by estrogen, by a direct action on the gonad (12). LCs synthesize and secrete testosterone for export and have paracrine actions on neighboring seminiferous epithelium, namely, in the initiation and maintenance of spermatogenesis (13). This effect is probably indirect because it has been reported that GCs themselves do not express a functional AR in adult humans. Therefore, androgen regulation is thought to be mediated by AR-expressing SCs and PCs (14). Interestingly, human adult testis LCs do express the AR weakly (14).

In the adult human, testis aromatase is localized to LCs and GCs (15). We have reported that aromatase mRNA is expressed in the prepubertal human testis, including the period of early postnatal activation (16), when local testosterone substrate production is high. Testicular estradiol, then, might have a role in the testis and in male reproductive tract development (17). Indeed, ERβ and ERα have been described in the human fetal testis, although expression of ERβ is several times higher than ERα, or the latter is not detected (18). In

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Abbreviations: AR, androgen receptor; **cP450$_{scc}$,** cytochrome P-450 side chain cleavage enzyme; **ER,** estrogen receptor; **GC,** germ cell; **GR,** group; **ICs,** interstitial cells; **LCs,** Leydig cells; **PCs,** peritubular cells; **SC,** Sertoli cells

1998, several variants of ERβ were reported (19,20), particularly, ERβcx, also named ERβ2, a truncated variant at the C-terminus, leading to the loss of 61 amino acids (21). Saunders *et al.* (21) reported that ERβ1 and ERβ2 are expressed in distinct cell populations in the adult human testis: ERβ1 is immunoexpressed more intensely in pachytene spermatocytes and round spermatids, whereas ERβ2 was found preferentially in SCs and spermatogonia. The existence of aromatase and ERs in various GCs suggests that estrogens are involved in human male gamete maturation (22). No information, however, is available on the expression of ERs in the human prepubertal testis.

In the male marmoset, it has been described that neonatal SCs, in contrast to adult SCs, might be targets primarily for estrogens rather than androgens (23). Indeed, during testicular postnatal activation and in early prepuberty, these authors reported a strong immunoexpression of ERβ, but not AR, in SCs and GCs of this nonhuman primate.

In this study, we report the expression of aromatase, ERα, ERβ, AR, and $cP450_{scc}$ in testes of human prepubertal subjects belonging to three developmental age groups. Among somatic cells of newborns and infants, ICs and LCs expressed aromatase preferentially. Estrogens so formed might interact with ERβ, but not ERα, located in ICs and SCs (particularly in newborns). Androgen, in turn, might interact with PCs and ICs, but not with SCs or LCs, during this period of life. Finally, GCs seem to function as a relatively independent system in terms of local responsiveness to sex hormones, expressing strongly aromatase and ERβ, but not ERα or AR.

MATERIALS AND METHODS

Clinical material. Human prepubertal testes were collected at necropsy from patients who died of disorders not related to endocrine or metabolic diseases. Following institutional rules, all necropsies were authorized by parents or relatives. Cadavers were placed at 4°C within 1 h after death. Necropsies were carried out within the following 12 h. In every case, written consent from the closest relatives had been obtained. The study was approved by the Institutional Review Board of the Garrahan Pediatric Hospital. Testes from 15 prepubertal subjects, aged 0.003 to 3 y old were studied. As previously described (4), samples were divided into three age GRs: GR1, newborns (1- to 21-d-old neonates, $n = 5$); GR2, postnatal activation stage (1- to 7-mo-old infants, $n = 6$); GR3, early childhood (12- to 60-mo-old boys, $n = 4$). Death was secondary to multiple diseases in every group of subjects, but congenital cardiac malformation was responsible for death in approximately 80% of cases of GR1 and in 40% of GR2. Pneumonia, sepsis, encephalopathy, and intestinal malformation were other diagnoses. A preparation from a control 25-y-old human adult male (provided by Centro de Investigaciones en Reproduccion, School of Medicine, University of Buenos Aires) was also used. Testes collected at necropsy were fixed in 4% formalin in phosphate-buffered saline, embedded in paraffin, immediately frozen, and stored in liquid nitrogen for subsequent RNA analysis or processed for cell isolation and culture.

Immunohistochemistry. Immunohistochemistry was performed employing the streptavidin-biotin and peroxidase method using the manufacturer's protocol [DAKO Catalyzed Signal Amplification (CSA) System; horseradish peroxidase; DAKO Cytomation, Carpinteria, CA]. Briefly, after deparaffinization, sections (5 μm) were subjected to antigen retrieval (30 min at 100°C in 10 mmol/L citrate buffer, pH 6.0). Endogenous peroxidase activity was quenched. The sections were further blocked with a protein block for 30 min to decrease nonspecific staining.

Sections were incubated with one of the following antibodies: against ERα, a monoclonal mouse antibody (4 μg/mL, sc-8002, Santa Cruz Biotechnology, Inc.); against ERβ, a goat antibody (4 μg/mL, sc-6820, Santa Cruz Biotechnology Inc.); against aromatase, a rabbit polyclonal antibody (1/500, Hauptman Woodward Institute, Inc., Buffalo, NY); against AR, a monoclonal mouse anti-human IgG (5 μg/mL, M3562 DAKO Cytomation); and against $cP450_{scc}$, a rabbit polyclonal antibody (1/200, AB 1244, Chemicon International, Temecula, CA) for 18 h at 4°C. After washing, tissues were incubated for 15 min with biotinylated goat anti-rabbit ($cP450_{scc}$ and aromatase), biotinylated rabbit anti-mouse (ERα and AR), or biotinylated rabbit anti-goat (ERβ) immunoglobulins, followed by the streptavidin-biotin complex, the amplification reagent, and the streptavidin-peroxidase conjugate. Bound antibodies were visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB), which results in a brown-colored precipitate at the antigen site. Counterstaining was performed using hematoxylin, which stains cell nuclei blue. As negative controls, normal rabbit serum (for $cP450_{scc}$ and aromatase) or normal mouse serum (for ERα and AR) or a normal goat serum (for ERβ) was used instead of primary antibodies. No specific immunoreactivity was detected in these sections. Experiments were repeated twice, and there were no differences in the patterns of immunolocalization between the two experiments. Human placenta (aromatase), human breast carcinoma (ERα and ERβ), and human prostate (AR) were used as positive controls.

To evaluate LCs, immunostaining and, to better recognize cell type, an additional preparation with combined hematoxylin-eosin staining were used. These double stainings introduced some loss of contrast. However, immunostained LCs could be clearly identified.

LCs were recognized by their large, polyhedral profile and eosinophilic cytoplasm. ICs also include mesenchymal cells, macrophages, LC precursors and fibroblast-like cells without the features described for LCs. Among GCs, gonocyte or primordial GC is large and has basophilic cytoplasm, sometimes found in the center of the seminiferous cords, isolated from the basement membrane by the supporting immature SCs. They represent a minority of GCs, present mostly in GR1. The second type of GCs is smaller and more irregular in shape and show attachment to basement membrane. It is the so-called transitional or type A primitive spermatogonia. Sometimes, some GCs appear swollen and bi- or multinucleated and show signs of cell death; they are called hypertrophic spermatogonia (1). As an additional means of identification, labeling with a c-kit antiserum was carried out. As expected, only GCs were labeled within the seminiferous cords (data not shown).

Positively stained cells were counted in single sections using a Carl Zeiss Axiovision microscope under a 100× objective lens. Approximately 300 ICs, PCs, and SCs or 100 in the case of GCs, per each slide, were counted. In the case of LCs, cell counting from different subjects of the same group was pooled to reach close to 100 total cells per group. For quantification, a modification of the proportionate score of Allred *et al.* (24) was used as follows: after the mean number of positive cells per 100 total cells was calculated per subject, a group mean was calculated (except for LCs as explained). A mean of <1% was considered as negative (−), >1 to 5 as ±, >5 to 20 as +, >20 to 35 as ++, >35 to 50 as +++, and >50% as ++++.

Detection of ERα, ERβ, and ERβ isoform mRNA expression by reverse-transcriptase polymerase chain reaction (RT-PCR). Total RNA was extracted from each testicular tissue by homogenization in TRIzol reagent (Invitrogen, Buenos Aires, Argentina) following the manufacturer's instructions. The integrity of each sample was checked by the presence of intact ethidium bromide-stained 28s and 18s ribosomal RNA bands. The purity of RNA samples was assessed by the 260/280 ratio (between 1.6 and 1.9) and by the absence of bands corresponding to contaminating DNA in the agarose electrophoresis. The RNA concentration was assessed by spectrophotometric absorbance at 260 nm.

Total RNA was reversely transcribed using Moloney murine leukemia virus RT (MuMLV-RT) (Amersham Biosciences. Buenos Aires, Argentina) following the manufacturer's instructions. Briefly, 5 μg of total RNA and 500 ng of oligo $(dT)_{15}$ primer (Biodynamics SRL, Buenos Aires, Argentina) were denatured by heating to 70% for 10 min, quickly chilled on ice, and subsequently incubated with 200 U of MuMLV-RT, 2.5 μL of fivefold concentrated RT reaction buffer, 1 mmol/L of each deoxyribonucleoside triphosphate (dNTP) (Promega, Buenos Aires, Argentina) and 25 U of porcine RNAguard Ribonuclease Inhibitor (Amersham Biosciences) in a 25 μL of reaction volume, at 37°C for 60 min.

The RT products were pooled and amplified by PCR. Primers located at the N-terminal A/B region of ERα and ERβ (19) were ERα sense 5′-aggctgcggcgttcggc-3′, antisense 5′-agccatacttcccttgtcat-3′; ERβ sense 5′-ttcccagcaatgtcactaact-3′, antisense 5′-ctctttgaacctggaccagta-3′. Primers located at the 3′ terminus of ERbeta1, 2 (5′-tgctgctggacgccctggc-3′, sense) and ERbeta1 (5′-tgtgggttctgggagccctc-3′; antisense) were for ERβ1 mRNA amplification. ERbeta1, 2, and ERbeta2 (5′-tgctccatcgttgcttcagg-3′; antisense) primers were for ERβ2 mRNA amplification. Each primer pair was localized on different exons to discriminate the products from genomic DNA and cDNA. PCR was carried out using 1 μL of cDNA pool as a template in 25 μL of reaction volume containing 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTP, 0.24 μmol/L of each forward and reverse primer, and 1 U *Taq* polymerase (Amersham Biosciences). Each reaction consisted of 35 cycles (1 min at

94°C, 1 min at each optimized annealing temperatures, 56°C for ERα and ERβ, 58°C for ERβ2, and 63°C for ERβ1 and 1 min at 72°C).

Negative controls lacking cDNA were included in all PCRs. Human ERα and ERβ cDNA clones (plasmids kindly supplied by K. Korach) and human placenta cDNA were used as positive controls. Specific oligonucleotide primers (25) were used for the amplification of 524 base pairs of a partial sequence of human β-actin mRNA. All samples were positive for β-actin mRNA.

PCR products were analyzed on 2% agarose gels containing ethidium bromide and visualized using a UV transilluminator. The identities of RT-PCR products were verified by sequencing analysis.

RESULTS

Table 1 summarizes the quantitative estimation of the immunoexpression of aromatase, ERβ, ERα, AR, and $cP450_{scc}$ in different somatic cell types of the testis for each prepubertal stage. Strong immunostaining of aromatase was detected in LCs in GR1 (Table 1 and Fig. 1*e*) and also in GR2, although of less magnitude. Important staining was observed in ICs in GR1 and GR2 (Table 1). Occasionally, staining of aromatase was found in ICs in GR3 (Table 1 and Fig. 1*a*, *inset*). Poor staining of ERβ was detected in LCs in GR1 (Table 1, Fig. 1*f*), while strong immunostaining was detected in ICs, PCs, and SCs in this group (Table 1 and Fig. 1*b*), followed by progressive decreases in staining intensity in GR2 and GR3. ERα was barely detectable in all cell types (Table 1 and Fig. 1*c*), including LCs (microphotographs not shown). This finding contrasted with the positive immunostaining detected in the breast tissue (Fig. 1*c*, *inset*). Table 1 and Figure 1*d* also show that the strongest expression of AR was detected in PCs of neonatal and infant testes. This included the second PC layer, which might be formed by peritubular mesenchymal cells. AR was also present in ICs and occasionally in LCs of GR1 and GR2. Remarkably, AR was absent in SCs in the three prepubertal groups (Table 1 and Fig. 1*d*), but it was present in SCs of an adult control testis (Fig. 1*d*, *inset*). Even though LCs were identified morphologically, $cP450_{scc}$ immunostaining helped to confirm their functionality (Table 1 and Fig. 1*h*) as well as to identify a small number of steroid-secreting cells among ICs and peritubular mesenchymal cells (Fig. 1*i*).

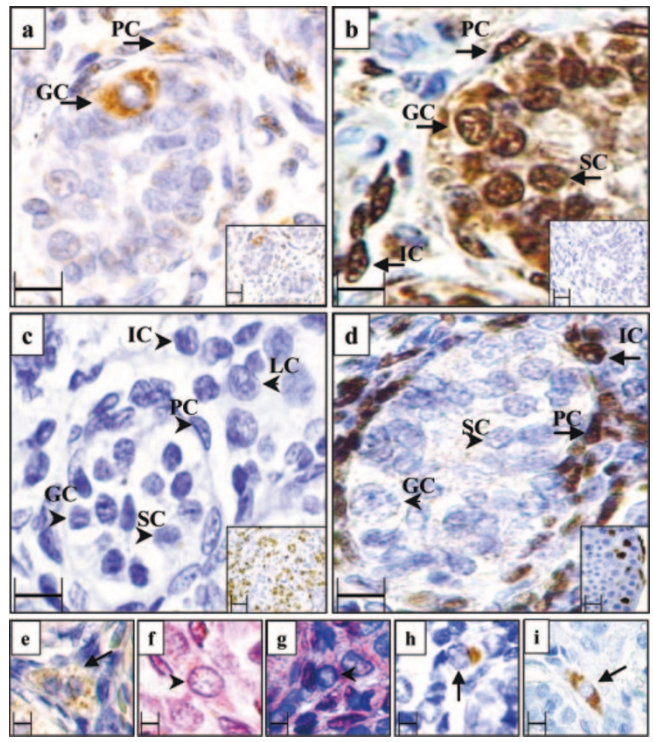

Figure 1. (*a*) Aromatase immunostaining in a testis of GR3. Strong cytoplasmic staining was seen in a GC and in a PC; *inset*: panoramic of the same preparation. (*b*) ERβ immunolabeling in a testis of GR1. Strong nuclear staining was present in ICs, PCs, SCs, and GCs; *inset*: negative control (normal serum). (*c*) ERα in a testis of GR1. No immunostaining was observed in any cell type, including LCs; *inset*: positive tissue control (breast tumor). (*d*) AR immunolabeling in a testis in GR1. Strong nuclear staining was present in PCs and ICs, but not in SCs or GCs; *inset*: positive SC control (SCs of an adult testis). Scale bar: 20 μm. (*e–h*) Immunocytochemistry of LCs in testes from GR1. (*e*) Aromatase-positive LC cytoplasm. (*f*) ERβ-negative LCs. (*g*) AR-negative LCs. (*h*) $P450_{scc}$-positive LCs. (*i*) $P450_{scc}$ immunocytochemistry of ICs in a testis from GR2. A positive cell with mesenchymal fibroblastic morphology, probably a LC precursor, is shown. Scale bar: 20 μm. *Thick arrows* point out to positively stained cells and *arrowheads* to negatively stained cells.

Table 1. *Immunohistochemistry cell localization and staining frequency of aromatase (ARO), ERβ, ERα, AR, and $cP450_{scc}$ enzyme in somatic cells and GCs of human prepubertal testes of three age groups*

	ARO	ERβ	ERα	AR	$cP450_{scc}$
ICs					
GR1 (n = 5)	++	+++	±	+++	+
GR2 (n = 6)	++	++	±	+	+
GR3 (n = 4)	+	+	±	+	±
LCs					
GR1 (n = 5)	++++	±	±	+	++++
GR2 (n = 6)	++	±	±	+	++++
PCs					
GR1 (n = 5)	+	++	−	++++	+
GR2 (n = 6)	+	+	±	+++	+
GR3 (n = 4)	+	±	±	+	−
SCs					
GR1 (n = 5)	±	+++	−	±	−
GR2 (n = 6)	±	+	−	±	−
GR3 (n = 4)	±	+	−	±	−
GCs					
GR1 (n = 5)	++	+++	−	±	−
GR2 (n = 6)	++	++	−	−	−
GR3 (n = 4)	++	++	−	−	−

Table 1 also summarizes the quantitative estimation of the immunoexpression of aromatase, ERβ, ERα, AR, and $cP450_{scc}$ in GCs (gonocytes and spermatogonia) of the testis, according to prepubertal stage. Aromatase (Fig. 1*a*) and ERβ (Fig. 1*b*) were expressed in GCs of the three age groups. No staining of ERα, AR, or $cP450_{scc}$ was detected in GCs.

RT-PCR was performed to evaluate the expression of ERα and ERβ mRNA in prepubertal human testicular tissue. Although ERα-specific PCR products were detected in cDNA prepared from human placenta (positive control) (25), no specific signal was present in the cDNA pool prepared from human testicular tissues of the three age groups (Fig. 2). In contrast, ERβ-specific cDNA was amplified from the same pool of testicular cDNA (Fig. 2).

ERα and ERβ specific primers span intron 1 of the poorly conserved N-terminal A/B region of the two respective ERs. These ERβ specific primers cross-react with wild-type ERβ (ERβ1) and with the ERβ2 receptor splice variant. Therefore, we then use C-terminal isotype specific primers to assess the

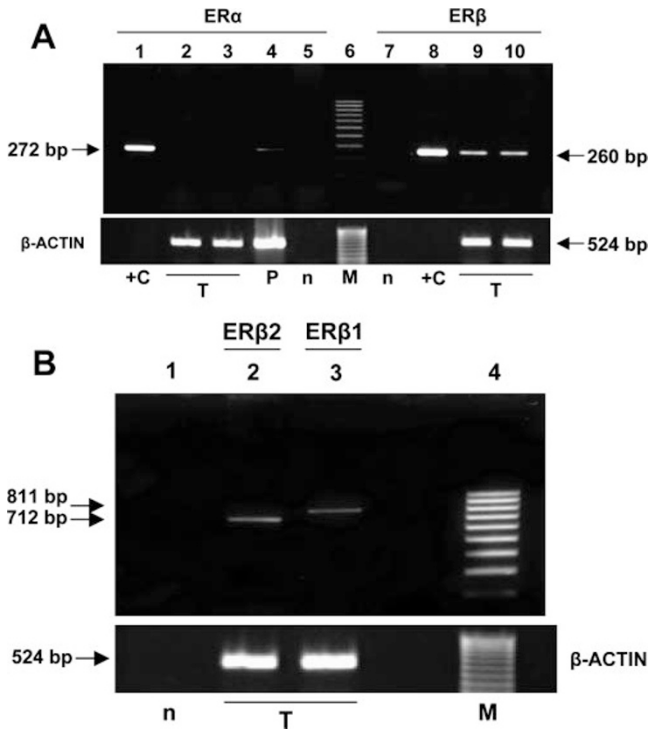

Figure 2. RT-PCR analysis of ERα, ERβ, ERβ1, and ERβ2 in human testicular tissue. (*A*) Expression of ERα (lanes 1–5) and ERβ (lanes 7–10): PCR amplification with primers specific for ERα (272 bp) and ERβ (260 bp) revealed that ERβ but not ERα mRNA was detected in a pool of human prepubertal testes (T). Control tissue [human placenta (P), lane 4] was positive for ERα. Human ERα and ERβ cDNA clones (lanes 1 and 8, respectively) were used as positive controls (+C). (*B*) Expression of ERβ1 (lane 3) and ERβ2 (lane 2): PCR performed with ERβ isotype-specific primers revealed that ERβ1 (811 bp) and ERβ2 (712 bp) mRNA were both expressed in a pool of human prepubertal testes (T). The 100-bp markers (M) were run in both gels; no product was amplified in reactions that did not contain cDNA (n).

presence of ERβ1 and ERβ2 isoforms. By RT-PCR, a single band corresponding to the expected size for ERβ1 (811 bp) and ERβ2 (712 bp) was amplified from the testicular cDNA pool (Fig. 2).

DISCUSSION

We found that aromatase is expressed in LCs of the early postnatal testis. Because LCs are the main androgen-producing cells, the enzyme might play a role in modulating testosterone secretion: the expression decreases when peak testosterone secretion occurs in boys in GR2.

The differentiation of mature LCs is believed to be derived from ICs or peritubular mesenchymal cells (26). We found that some of these cells exhibit positive $cP450_{scc}$ staining, suggesting that they have steroidogenic capacity and could be considered as precursor LCs. Reported evidence suggests that estrogen inhibits LC development and function, based on studies in rodents (27). Although these effects could be indirect by inhibiting gonadotropic secretion, the presence of aromatase and ERs in LCs suggests that estrogen could act directly on these cells. However, considerable species variations and type of ER expression have been published. For instance, it has been proposed that endogenous estrogen inhibits mouse fetal LC development *via* ERα (28). The importance of this report for the human is at least questionable, in view of the poor expression of ERα reported in human fetal testis (29) and of our findings in the postnatal testis, in this study.

We found that ERβ is the predominant form of ER expressed in ICs, PCs, and SCs of neonates and infants. This was confirmed by RT-PCR of both ERα and ERβ mRNA. Saunders *et al.* (30) reported widespread expression of ERβ in the primate adult male reproductive system. However, later studies found that in adult SCs and spermatogonia, ERβ2, an isoform that lacks estradiol binding and that may act as a dominant negative inhibitor of ER action, was preferentially detected in these cells (24). Our ERβ antibody does not differentiate ERβ1 from ERβ2. Therefore, by specific RT-PCR, we looked for the presence of ERβ1 and ERβ2 mRNAs and found that the two ERβ mRNA isoforms are expressed in the postnatal human testis.

The local effect of estrogen in the testis is not well defined. The information available includes mostly inhibitory effects (31), such as inhibition of testosterone production under gonadotropic stimulation (12), although stimulatory actions have also been reported. For example, estrogen induces spermatogenesis in the hypogonadal mouse (32) and act as a germ survival factor in the human testis *in vitro* (33). In summary, the role of estrogen on maturation and proliferation of the prepubertal human testis is complex and requires further studies.

PCs expressed AR at high percentages and with intense immunostaining, particularly in newborns and infants. Two types of PCs have been described. Peritubular myoid cells have been shown to be contractile and to secrete a number of substances including extracellular matrix components and growth factors (34). Some of these substances have been proposed to affect SC function, such as PModS. However, the identity of PModS remains elusive, and its effects are mimicked by a number of growth factors (35). Peritubular mesenchymal cells, on the other hand, have been proposed to give rise to precursor LCs (27), and, indeed, as mentioned above, we have detected expression of a steroidogenic enzyme in fibroblast-like ICs. We have detected the AR in more than one layer of PCs, suggesting that it might be present in the two types of PCs. It is possible that androgens, secreted by the remaining fetal LCs, on interacting with the AR of precursor LC fibroblasts of newborns (GR1) and infants (GR2), might modulate, along with other factors such as estrogen and the insulin-like growth factor (IGF) system, the proliferation, migration, and differentiation of new infantile LCs (36).

Our finding of poor expression of AR in prepubertal SCs is interesting because it is in contrast with the strong expression reported in adult SCs (14). The absence of AR expression in the human prepubertal SCs contributes to explaining why no GC development is seen in normal infants during the postnatal activation of androgen secretion by the testis. Lack of spermatogenic development in young infants probably favors the preservation of an adequate pool of GCs for future fertility in adulthood.

GCs present in the prepubertal human testis are gonocytes (mainly in neonates) and primitive type A spermatogonia. In the rat, the expression of aromatase is threefold higher in pachytene spermatocytes compared with gonocytes (15). In the prepubertal human male, we report active expression of both aromatase and ERβ in GCs, indicating that estrogens can be synthesized in GCs from androgens provided by LCs and precursor ICs, to have an effect in the same GCs and/or in neighboring SCs. Similar to what has been described in more mature GCs (14), we have not detected expression of AR in immature GCs, indicating that any effect of androgens on these cells must be mediated by indirect mechanisms.

In summary, we propose that local production of testosterone by steroidogenic precursor cells or remaining fetal LCs, probably acting through PCs or ICs, might be one of the factors involved in the induction of infantile LC differentiation, whereas the role of estrogen is less clear, but it probably modulates ICs, precursor LCs, and GC mass and function during human prepuberty.

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