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Human foetal testis: source of estrogen and target of estrogen action

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ABSTRACT:

Background: Estrogens are involved in masculine fertility and spermatogenesis. However, little is known about estrogen involvement in human testicular organogenesis.

Objective: Analysis of cellular sources and targets of estrogens and of their variations in the human testis during foetal development. Expression profiles of aromatase (CYP19) and estrogen receptors α and β were analyzed in human foetal testes at various gestational stages by immunohistochemistry and quantitative RT-PCR

Materials: Fifty-four archival paraffin-embedded and 4 frozen foetal testes were studied by immunohistochemistry and real time PCR. Tissue quality was confirmed by histology and expression of specific functional markers: androgenic enzymes for Leydig cells, anti-Müllerian hormone for Sertoli cells and Steel factor receptor for germ cells.

Results: We demonstrate that human foetal testes express aromatase and ERβ simultaneously in Sertoli, Leydig and germ cells and are devoid of ERα. Quantification of positive cells indicates a window of protein expression, especially between 13 and 22-24 weeks. Quantitative RT-PCR confirms that the human foetal testis expresses CYP19 and ERβ but not ERα messenger RNA.

Conclusion: Our findings suggest that locally produced estrogens influence human testicular development through autocrine and paracrine mechanisms, most notably during the period of maximal testicular susceptibility to endocrine disruptors.

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INTRODUCTION

Estrogens have been traditionally considered female hormones but are physiologically important in both sexes and target several male organs (Hess, 2003; Nilsson and Gustafsson, 2002). Estrogen action is mainly mediated by activation of two specific receptors (ER) in target cells: ERα and ERβ, highly homologous ligand-inducible transcription factors regulating the expression of specific genes. The synthesis of estrogens from androgenic precursors is catalyzed by the aromatase enzyme complex situated in the endoplasmic reticulum of estrogen-producing cells. The human aromatase is the product of the 18-exons CYP19 gene localized in the 15q21.1 region. CYP19 gene expression in man is regulated by different tissue-specific promoters upstream of unique first exon encoding 5'-untranslated regions of the aromatase mRNAs, but the 503 amino acids translated protein is the same in all tissues. In the human male the enzyme is expressed in testis, adipose tissue, skin, and brain and also in various foetal tissues (Simpson et al., 1994). Estrogen concentrations in the *rete testis* fluid of various adult male species including the human are higher than in plasma, indicating the occurrence of testicular estrogen synthesis (Hess, 2003; O'Donnell et al., 2001). Mature Leydig cells are the main source of testicular estrogens in mammals (Hess, 2003),while Sertoli cells of adult men do not express aromatase in significant quantities (Brodie et al., 2001) (Turner et al., 2002). Conversely, in neonatal and prepubertal animals immature Sertoli cells are a source of estrogens, Sertoli cells aromatase expression declines during maturation (Payne and Youngblood, 1995; Sharpe et al., 2003). Recently, the ability of aromatization of germ cells and sperm has been demonstrated in various species including the human. These cells are an additional source of estrogens in the adult testis (Carreau et al., 2003).

Besides being capable of estrogen synthesis, adult testes are also targets of estrogen action. The presence of ER has been well documented in adult testes. ERβ have been detected in rat

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and mouse Leydig, Sertoli and germ cells at various stages of maturation. The presence of ERα has been reported in rodent Leydig cells (Fisher et al., 1997). ERβ, but not ERα have been described in Sertoli, Leydig, and germ cells of mature nonhuman primates (Sierens et al., 2005), and in the adult man (Hess, 2003; O'Donnell et al., 2001). The results of immunolocalization studies of ERα in the adult human testis are conflicting. This receptor, if present, seems restricted to Leydig cells (Pelletier and El-Alfy, 2000).

Few studies are available on ER expression in the foetal testis. In the rodent, ERβ is expressed in foetal Leydig, Sertoli and germ cells and ERα in interstitial cells. In rare studies concerning the human foetal testis, ERβ is detected in germ, Sertoli and interstitial cells between 13 and 20 weeks of development but ERα is absent (Gaskell et al., 2003; Sierens et al., 2005; Takeyama et al., 2001).

While estrogen effects on female reproduction are traditionally well known, their actions on the testis have been only recently elucidated after studies on genetically modified mice lacking functional ERα (αERKO) or ERβ (βERKO) (Korach, 1994) or with disruption of the CYP19 gene (ArKO) (Fisher et al., 1998), (Robertson et al., 1999). Case studies of men with inactivating mutations of ERα or CYP19 genes also suggest a role of estrogens in human masculine fertility (Carani et al., 1997; Morishima et al., 1995; Smith et al., 1994). Few data exist on estrogenic effects on human masculine gonadogenesis. The ability of the human foetal testis to aromatize androgenic precursors is also unclear. We thus decided to examine the expression of both ER and aromatase proteins and mRNAs in the human foetal testis up to the final stages of development to determine whether the human foetal testis is capable of estrogen production as the adult gonad. We also wished to identify the cellular targets of the locally produced hormones and the temporal relationship between susceptibility to hormone action, estrogen production and stages of organogenesis. The use of immunohistochemistry with specific antibodies allowed us to provide evidence for the capacity of estrogen synthesis

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of the human foetal gonad and to identify the cellular subpopulations involved. We also detected the cellular targets of estrogens and their variations during testicular development. Our immunohistochemical results on protein expression were confirmed by the finding of the corresponding RNA messengers by real time PCR.

MATERIALS AND METHODS

Tissues

Fifty-four archival formol-fixed paraffin-embedded foetal testes were selected from the organ bank of the Pathology Institute of Bari's University after approval of the local ethical committee. Testes from foetuses with abnormal karyotypes or macroscopic and histological abnormalities were excluded. Normal adult human testes, cycling ovaries, endometria and porcine ovaries were used as positive controls.

Snap-frozen testes from 13, 24, 25 and 35 week-foetuses obtained from the tissue bank of the Pathology Institute of Bari's University were used for real time quantitative RT-PCR. Normal frozen human placenta, adult testes and cycling endometria were used as controls. The human samples were from a declared tissue collection according to French bioethical law n° 2004-800.

Antibodies

Eight different antibodies were used: polyclonal anti-P450scc (P450 side chain cleavage) (Hanukoglu et al., 1990), anti-3βHSD (3-Beta-hydroxysteroid-dehydrogenase) (Dupont et al., 1990)anti-P450c17α (P450c17 alpha hydroxylase) (Kominami et al., 1983), anti-aromatase (Osawa et al., 1987) anti-Anti-Müllerian Hormone (AMH) (Kind gifts respectively of Dr I Hanukoglu, College of Judea and Samaria, Israel, of Pr Van-Luu The, Laval University, Canada, of Pr S Takemori, Hiroshima University, Japan, of Pr Y Osawa, Michigan

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University, USA, and of Dr N Di Clemente INSERM U 493 Clamart, France) anti-c-kit (DakoCytomation, Glostrup, Danemark) at the 1:8000, 1:3000, 1:8000, 1:700, 1:4000, 1:200 dilutions respectively, and monoclonal anti-ERα (clone 1D5 DakoCytomation) and anti-ERβ, directed against the β1 isoform (MCA 1974, Serotec LTD. Oxford UK) at the 1:20 and 1:40 dilutions respectively.

Immunohistochemistry

Briefly, 5µm-thick tissue sections were deparaffinized and rehydrated by successive baths of toluene and graded alcohols and subjected to 15 min microwave antigen retrieval in pH 6 citrate buffer. After cooling and 15 min pre-incubation with a blocking serum (DakoCytomation), slides were incubated with the primary antibodies overnight at 4°C in a humid chamber. Bound immunoglobulins were revealed with a streptavidin-biotin-peroxydase-aminoethylcarbazole kit (LSAB+, DakoCytomation) according to manufacturer's instructions.

Counting of immunostained cells

At least six 40x power fields were digitized for each sample with an 18.2 Spot software camera (Diagnostic Instruments Inc. MI, USA). The digital images were transferred on a computer screen and a grid was superimposed using Adobe Photoshop CS2 software. The percentage of stained cells and the intensity of staining of each testicular subpopulation were evaluated blindly by two independent observers.

Real time RT-PCR

Specific gene expression was quantified by real time PCR. Total RNA was isolated from frozen samples by TRIZOL reagent (Invitrogen) according to the manufacturer's

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recommendations. RNA concentration was determined by measuring absorbance at A_{260} and A_{280} with a ratio of 1.8 or higher. The integrity of RNA was visualized on agarose gel. Two µg of total RNA were subjected to DNase I treatment (DNase I Amplification Grade procedure (Invitrogen)) and then reverse transcribed with 200 units of reverse transcriptase using random hexamer primers (Superscript kit). PCRs were performed with 200ng cDNA in the presence of qPCR™ Mastermix Plus for Sybr™ Green I (Eurogentec, Seraing, Belgium) containing 300nM of specific primers (Table 1). Real time PCR was carried out on ABI 7000 Sequence Detector (Applied Biosystems, Foster City, CA). For standards preparation, amplicons were subcloned into pGEMT-easy plasmid (Invitrogen) and sequenced to confirm the identity of each fragment. Standards curves were generated using serial dilutions of linearized standard plasmids. All samples and standards were amplified in duplicate. Amplification of ribosomal 18S was used as internal control for data normalization. Results are mean ± SEM of at least two independent analyses of at least two different reverse transcribed samples. Relative expression of a given gene is expressed as the ratio (attomoles of specific gene/femtomole of 18S).

RESULTS

Verification of the integrity of tissue samples

The selected foetal testes had a normal morphology with seminiferous tubules constituted by Sertoli and germ cells separated by interstitial tissue including foetal Leydig cells with round nuclei and abundant cytoplasm and peritubular elongated Leydig cell precursors.

As a further validation of the integrity of the foetal testes, each sample was studied by immunocytochemistry with well-characterized antibodies recognizing specific markers of Leydig, Sertoli and germ cells.

The immunoexpression pattern of the steroidogenic enzymes involved in androgen synthesis

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P450scc, 3βHSD and P450c17α (Murray et al., 2000) was used to examine the foetal Leydig cell population. Foetal Sertoli cells integrity was estimated by expression of AMH, functional marker of Sertoli cell differentiation responsible of Mullerian ducts regression produced from the 8th week of gestation (Rajpert-De Meyts et al., 1999). C-kit, the Steel factor receptor was used to evaluate germ cells (Robinson et al., 2001). All markers were evaluated semiquantitatively. Samples lacking one or more markers or with an abnormal quantity of immunopositive cells for the foetal age (Murray et al., 2000), (Habert et al., 2001) were excluded from the study.

Immunolabeling for the steroidogenic enzymes P450scc (Fig 1 A, B, C; D), 3βHSD (Fig 1 E, F, G, H) P450c17α (Fig 2 A, B, C, D) was always localized in the cytoplasm of interstitial cells. Two types of immunopositive cells could be distinguished: polygonal, with round nuclei and abundant cytoplasm corresponding to foetal Leydig cells and peritubular, elongated Leydig cell precursors with smaller nuclei and scanty cytoplasm. Immunolabelling intensity was constant throughout foetal testicular development but the number of immunopositive cells varied (Fig 3A). Fifty per cent of interstitial cells were labelled at the fourteenth week of foetal life, (Fig 2A) their number peaked around the 18- 19th week (Fig 2B) to decrease afterwards. The labelled interstitial cells never completely disappeared, however, at 28-35 weeks of age only few immunopositive cells could be observed in a reduced interstitial space between the seminiferous tubules (Fig 2D). The staining intensity was the same.

AMH expression was localized to the cytoplasm of intratubular Sertoli cells and constantly found at all foetal ages examined (Fig 2 E, F, G, H and Fig 3). Germ and Sertoli cells represented about 25% and 65% of the intratubular population respectively. The counting was performed according to nuclear morphology as the proximity of the cells made it difficult to discriminate the two populations by AMH expression only. C-kit immunoexpression was limited to the membrane of germ cells (Fig 1, I, J, K, L, M). About 25% of the germ cells

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were immunonegative. This marker was constantly expressed during testicular development, however the number of C-kit positive cells was more abundant between 18 and 22 weeks of age (Fig 1 J,K) and decreased at the end of term (Fig 1L).

Aromatase expression

Aromatase immunolabelling was localized to the cytoplasm of both interstitial and intratubular cells. Labelled interstitial cells (Fig 2 I, J, K, L and Fig 3) had round nuclei and polygonal shape or an elongated appearance. The enzyme could be localized in 50% of the interstitial cells of foetal testes from the 13-14th week of life (Fig 2I and Fig 3A). Interstitial aromatase expression increased gradually: 65% of interstitial cells were labelled up to the 18-20th week of foetal life (Fig 2J and Fig 3A). The number of immunopositive cells started decreasing afterwards. At 22 weeks of foetal development, only 50% of the interstitial cells were immunopositive (Fig 2K, Fig 3A) while at 35 weeks aromatase expression ceased almost completely (Fig 2L). In the intratubular spaces both Sertoli and germ cells were immunolabelled (Fig 2I and Fig 3B), the enzyme was expressed in 50% of the intratubular cells at 14 weeks, in the majority of intratubular cells at 17-18 weeks and decreased gradually between 18 and 22 weeks (Fig 2K), and more abruptly afterwards. No immunopositive cells were visible at 35 weeks of age (Fig 2L and Fig 3B).

ERβ and ERα expression

ERβ immunolabelling was localized to the nuclei of both interstitial and intratubular cells (Fig 2 M, N, O, P and Fig 3). In the interstitium, cells with abundant clear cytoplasm and round nuclei and peritubular cells with an elongated nucleus and scanty cytoplasm were immunopositive. In the intratubular spaces, both Sertoli and germ cells expressed the receptor. The expression (staining intensity and percentage of labelled cells) was almost constant

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between 14 and 22 weeks of foetal life, and involved about 50% of the interstitial (Fig 3A) and intratubular cells (Fig 3B). After 22 weeks of age the immunolabelling declined progressively to disappear completely near term.

In sharp contrast with ERβ, immunolabelling for ERα was constantly negative, in every cell of all foetal testes examined from the 13th to the 35th week of development (data not shown).

CYP19, ERβ and ERα mRNA

In order to get a confirmation of the presence of CYP19 and ER RNA messengers (mRNA) during foetal testis development, mRNA levels of these genes have been determined in 4 foetal testes at 12, 24, 25 and 35 weeks of age and in two adult (young and aged) testes using real time PCR (Fig 4).

Setting up of standard plasmids allows us to generate standard curves yielding correlation coefficients > 0.99 and efficiencies of at least 0.91 in all experiments. When real time PCR products were visualized on 3% agarose gel, 150bp amplicons for CYP19 (Fig 4A) and ERβ (Fig 4C) were detected in all the samples of foetal and adult testes.

In the human foetal testes analyzed the relative expression level of CYP19 mRNA was in the same range than that of the aged adult testis but lower than that of the young adult testis and relatively invariable at any age examined (Fig 4B). ERβ expression was more pronounced in the foetal gonads of 24 and 25 weeks gestational age than in the 12 and 35 weeks old testes (Fig 4D).

No transcript for ERα was found in any of the gonads studied while the messenger was highly expressed in the endometrium used as positive control (data not shown). These results indicate that ERα messengers are not expressed in the human foetal testis consistent with the absence of immunodetectable ERα .

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Although the number of our samples is limited due to the rarity of available frozen foetal testicular samples of good quality, our results clearly demonstrate that CYP19 and ERβ but not ERα mRNAs are expressed in the human foetal testis.

DISCUSSION

We demonstrate for the first time by immunohistochemistry and confirm by quantitative RT PCR the presence of protein and mRNA of aromatase in the human foetal testis. Aromatase immunoexpression is observed in foetal Leydig, Sertoli and germ cells from the 13th week of foetal life, to decrease after the 22nd week. The messenger is found from the 12th to 35th week. We also extend previous reports on ERβ localisation in the human foetal testis between 13 and 20 weeks (Gaskell et al., 2003; Takeyama et al., 2001) including in our study stages of foetal development up to the 35th week of gestation. The presence of the receptor protein is observed from the 14th to the 22nd week of gestation, while the messenger RNA is detected in samples from the 12th to the 35th week. ERα protein and transcript are not observed in all foetal testes examined.

Our results confirm that the human foetal testis is a target of estrogen action through the preferential activation of the β receptor particularly between the 13th and 22nd week. The immunoexpression of both aromatase and ERβ follows an almost parallel course. In line with previous reports (Murray et al., 2000), we detected a maximal immunoexpression of the enzymes involved in androgen synthesis in Leydig cells between 13 and 22 weeks of gestation. The contemporary high expression of aromatase suggests a significant local estrogen production through aromatization of the available elevated quantity of androgenic precursors. We also observed a simultaneous abundant expression of ERβ in human foetal germ, Sertoli and Leydig cells. Taken together, these findings suggest direct autocrine and paracrine effects of locally produced estrogens on the human foetal testis mediated through

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activation of the β receptor, especially between 13 and 22 weeks of development.

The window of expression of aromatase suggests a direct or indirect regulation of this enzyme by gonadotropic hormones. FSH has been shown to regulate aromatase levels in the testis of the prepubertal mouse and rat (Dorrington et al., 1978) while FSH and cyclic AMP stimulate aromatase activity in the foetal rat testis (Weniger and Zeis, 1988). LH-induced testosterone enhances CYP19 gene expression in Leydig and germ cells (Bourguiba et al., 2003).

Estrogen action on the adult testis has been the subject of many studies on laboratory animals and some reports about human patients. Studies of genetically modified rodents have demonstrated a positive effect of endogenous estrogens on testicular function. αERKO but not βERKO males are infertile through a defect of efferent ducts fluid reabsorption (Hess et al., 1997) (Korach, 1994). ArKO males, initially fertile, develop progressive infertility with spermatogenesis arrest (Fisher et al., 1998). While phenotypes of αERKO and ArKO males are indicative of critical roles of estrogens on fertility in adult rodents, mediated through ERα activation, the functions of estrogens on human masculine fertility are not completely elucidated. The limited observations on testicular function in adult humans with a disruption of the ERα gene (Smith et al., 1994) or incapable of estrogen synthesis secondary to congenital aromatase deficiency (Carani et al., 1997; Morishima et al., 1995), have not given clear-cut indications. The available data however suggest that estrogens are required for normal fertility in man.

Reports about the actions of endogenous estrogens on the foetal testis are rare. So far most studies on the estrogenic effects on testicular organogenesis are based on the administration of large doses of exogenous estrogens or estrogen-like compounds to animals and show a deleterious effect on fertility, with a decrease of testicular growth and sperm production. Some hypotheses can be formulated about the physiological roles of endogenous estrogens on human testicular development. During testicular organogenesis the number of foetal Leydig,

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Sertoli and germ cells is strictly regulated through a balance of proliferative and apoptotic stimuli (Habert et al., 2001). Proliferation in the human foetal testis is strong at 13-14 weeks and declines at 18-19 weeks (Murray et al., 2000) while apoptotic cells are rare at week 16, increase at mid gestation and persist till term (Ketola et al., 2003).

Human Leydig cells start differentiating from mesenchymal precursors at 8 weeks of age, proliferate between the 12th and 18th week and decrease by apoptosis after the 19-20th week of foetal life till only rare cells remain at the end of term (Murray et al., 2000), (Habert et al., 2001). Estrogens block the proliferation of precursor Leydig cells (Abney and Myers, 1991) and modulate Leydig cell steroidogenesis (Hsueh et al., 1978). Between the 13th and 22nd week of gestation locally produced estrogens might thus participate to a regulatory loop controlling precursor Leydig cell proliferation and differentiation thereby regulating testosterone synthesis, possibly through changes in Leydig cell responsiveness to LH (Huhtaniemi et al., 1980).

Sertoli cell growth in the human follows two proliferation waves, during testicular development and before puberty (Plant and Marshall, 2001). The number of Sertoli cells in the human foetal testis decreases with gestational age (Helal et al., 2002). After a period of proliferation, Sertoli cells divide less and, from the second trimester to term, decrease by apoptosis (Ketola et al., 2003). While high doses of estrogens are deleterious, low doses increase Sertoli cell proliferation (Atanassova et al., 2005). Estrogens also enhance immature Sertoli cell biosynthesis of adhesion proteins such as N-Cadherin (MacCalman et al., 1997) and increase ERβ expression in the mouse Sertoli cell line SK11 (Sneddon et al., 2005). Thus estrogens could induce foetal Sertoli cell proliferation, at the same time upregulating ERβ expression and enhancing Sertoli-germ cell adhesion. The cessation of estrogen production in the human foetal testis coincides with the observed decrease of Sertoli cells.

The effects of estrogens on spermatogenesis have been reported in several contradictory

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studies. In cultures of whole testis explants estrogens exert deleterious effects on perinatal mouse germ cells through ERβ activation (Delbes et al., 2004). However, estrogens induce proliferation of immature rat testis gonocytes (Li et al., 1997), at low concentrations inhibit adult human male germ cell apoptosis (Huhtaniemi et al., 1980) and act synergistically with FSH to increase spermatogenesis in the immature rat testis (Kula et al., 2001). Endogenous estrogens could therefore directly stimulate gonocyte proliferation in association with gonadotropins or local factors. At cessation of local estrogen effects, germ cell apoptosis would increase and proliferation decrease. In mice germ cell apoptosis is a requirement for fertility: failure to maintain a proper ratio between Sertoli and germ cells causes infertility (Rodriguez et al., 1997; Russell et al., 2002)

A higher level of complexity of estrogenic effects on foetal germ cells is added by previous findings on the differential distribution in the human foetal testis of two spliced variants of ERβ: β1 and β2 with different hormone affinity; the ERβ2 isoform seems to act as a suppressor of ERα and ERβ1-induced transactivation. The ERβ2 isoform predominates in foetal gonocytes between 13 and 18 weeks and could prevent estrogen action on foetal germ cells (Gaskell et al., 2003). Further studies about the presence of the variant forms in the human foetal testis at late stages of development and their relationship to aromatase expression are warranted to clarify this topic. Recent studies have also reported that in different tissues ERβ does not necessarily require estrogen as a ligand but can be activated, through binding to an ERE, by the short lived androgen metabolite 5-alpha-androstane-3-beta-17-beta-diol (3-beta-diol) (Pak et al., 2005; Picciarelli-Lima et al., 2006; Weihua et al., 2002). This alternative role of ERβ as a mediator of androgenic action suggests a possible synergic effect of locally produced estrogens and androgens on testicular organogenesis mediated by this receptor between 13-14 and 22-25 weeks. This hypothesis would confirm the particular importance of the β rather than the α estrogen receptor in the human masculine gonad.

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Concluding, our results individuate an important intratesticular production of Aromatase during foetal life and confirm the importance of estrogens on masculine fertility starting early during organogenesis. The main cellular subpopulations of the human foetal testis are both source and target of estrogenic action through ERβ activation especially between 13 and 22-24 weeks of development. The period of maximal estrogen synthesis and expression of the receptor suggests a modulating action on the effects of gonadotropins and locally produced hormones and growth factors on the proliferation and differentiation of Leydig, Sertoli and germ cells. Local estrogen action on the developing human testis could be a prerequisite for normal gonadal function in man.

The exclusive expression of ERβ in the developing human testis throughout gestation underlines an important difference between rodents, in whom ERα is needed for testicular development, and man, and suggests caution in extrapolating results in rodent models to human pathophysiology. A better comprehension of hormone effects on human testicular development could elucidate some antenatal causes of masculine infertility. The discovery of a window of maximal testicular susceptibility to estrogens could also have important implications in the studies of the effects of endocrine disruptors on human testicular development and function.

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Name	Accession	Amplicon	Sense primer	Antisense primer
18S	AJ844646	70 bp	GTGCATGGCCGTTCTTAGTTG	CATGCCAGAGTCTCGTTCGTT
ERα	AY425004	150 bp	TGCTGGCTACATCATCTCGGT	GACTCGGTGGATATGGTCCTTC
ERβ	AY785359	150 bp	CTAACTTGGAAGGTGGGCCTG	AGCGATCTTGCTTCACACCA
CYP19	BC056258	150 bp	CTAAATTGCCCCCTCTGAGGT	CCACACCAAGAGAAAAAGGCC

Table 1: Primer sequences of genes analyzed in Real Time PCR

The abbreviations of the genes, their GENBANK accession number and 5' to 3' nucleotide sequences of the sense and antisense primers are presented.

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LEGENDS OF FIGURES

Figure 1: Expression of the steroidogenic enzymes P450scc and 3βHSD and of the receptor C-kit the human foetal testis at 14, 18, 22 and 35 weeks of age respectively.

A, B, C, D: Expression of P450scc in the foetal testis. A cytoplasmic expression of the enzyme is visible in interstitial foetal Leydig cells (LC). The immunopositive cells are especially abundant at 14 and 18 weeks (A, B) and decrease at 22 weeks of age. The Sertoli and germ cells in the seminiferous tubules (ST) are negative.

E, F, G, H: Expression of 3βHSD in the foetal testis. The enzyme is visible in the cytoplasm of foetal Leydig cells (LC). The immunopositive cells are more abundant at 14 and 18 weeks of age (E, F) and decrease afterwards.

I, J, K, L: Expression of the receptor C-Kit in the foetal testis. The expression of C-Kit is evident on germ cells membranes in the seminiferous tubes (ST). The labeled cells are more numerous around 18-22 weeks of age (J, K) and decrease at the end of term (L).

Figure 2: Immunoexpression of P450c17α, AMH, aromatase and ERβ in human foetal testes at different developmental stages.

A, B, C, D: Expression of P450c17α in foetal testes. A cytoplasmic expression of the enzyme is visible in interstitial foetal Leydig cells (LC) and Leydig precursors. The immunopositive cells are abundant at the 14^{th} (A) and 18^{th} week (B) and decrease afterwards from 22 weeks of age (C). Only few immunopositive cells are visible at 35 weeks of age (D). Sertoli and germ cells in the seminiferous tubules (ST) are negative.

E, F, G, H: Immunolabelling with anti-AMH antibody: intratubular foetal Sertoli cells are strongly immunopositive throughout foetal development; germ cells and interstitial cells are negative. ST: seminiferous tubule

I, J, K, L: Aromatase immunoexpression. The enzyme is expressed in the cytoplasm of

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intratubular (ST) Sertoli and germ cells, and in interstitial foetal Leydig cells (LC) both at the 14^{th} ,18^{th} and 22^{nd} week (I, J, K). Inset: higher magnification of immunopositive Sertoli cells (arrows) in a 14 week old foetal testis (I). Note the complete disappearance of the enzyme at the 35^{th} week of gestation, both in the tubules (ST) and Leydig cells (LC) (L).

A light haematoxylin counterstain was performed in L.

M, N, O, P: Nuclear expression of ERβ in intratubular Sertoli and germ cells, and in interstitial foetal Leydig cells and precursors both at the 14^{th} ,18^{th} and 22^{nd} week(M, N, O). The immunolabelling decreases at 22 weeks (O) and disappears at the 35^{th} week of gestation (P). A light haematoxylin counterstain was performed in P to allow a better visualization.

Original magnification: x 40. Inset: x 100.

Figure 3: Evolution of aromatase, ERβ, AMH and P450c17α expression in human testis during fetal development

The percentage of stained cells for each marker was determined by two independent observers.

A: percentage of interstitial cells stained with aromatase, ERβ and P450c17α

B: percentage of intratubular immunopositive cells stained with aromatase, ERβ and AMH.

Data for each age represent mean ± SEM of at least 6 different fields. For each age bracket 1000 to 2200 intratubular cells and 1300 to 3000 interstitial cells were quantified.

Figure 4: CYP19 and ERβ messengers expression and quantification in human fetal and adult testes determined by real time PCR.

CYP19 (A-B) and ERβ (C-D) expression was studied by RT-PCR analysis.

A and C: 10µl of real time PCR reaction were visualized on 3% agarose gel.

Lanes 1 to 4: human foetal testes of 12, 24, 25 and 35 weeks of age respectively. Lanes A and

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B: two different samples of adult human testis. RT- (omission of the reverse transcriptase) and H_2O were negative controls.

B and D: Relative expression of CYP19 (B) and ERβ (D) mRNA in the human testis. Results were normalized by the amplification of the 18S ribosomal RNA. Data represents mean ± SEM of two different RT samples.

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