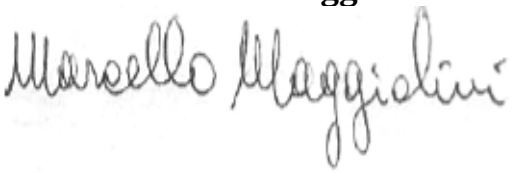
University of Calabria

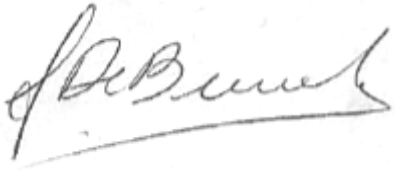
Ph.D. in Molecular Bio-pathology

Molecular mechanisms determining aromatase overexpression and inducing rat tumor Leydig cell proliferation: involvement of IGF-I and COX-2

Supervisor
Prof. Marcello Maggiolini



Coordinator
Prof. Giovanna De Benedictis



Candidate
Adele Chimento

2007

INDEX

.

Scientific Publications

IGF-I regulating aromatase expression through SF-1, supports estrogen dependent tumor Leydig cell proliferation.

Summary

Several studies on both humans and rodents indicate that prenatal or postnatal exposure to estrogens migth have a central role in the mechanism leading to male reproductive tract malformations as well as testicular tumors (1;2). While the effects of estrogen on mammary gland tumorogenesis is well known, the role of aromatase overexpression and *in situ* estrogen production in testicular tumorogenesis is not clearly defined. In this study we have investigated the molecular mechanisms causing aromatase overexpression and the effect of estradiol (E2) overproduction on Leydig cell tumor proliferation. Our hypotesis is that constitutive E2 production stimulates Leydig tumor cell proliferation acting on cell cycle regulators. Moreover, among several potential factors inducing aromatase, we investigated the role of IGF-I, produced locally in the testis, and of COX-2 overexpressed in other estrogen-dependent tumors. We used rat R2C Leydig tumor cells and testicular samples from Fischer rats with a developed Leydig tumor (FRTT). Both experimental models express high levels of aromatase and Estrogen Receptor alpha (ERα). Treatment with exogenous E2 induced proliferation of R2C cells and upregulation of cell cycle regulators cyclin D1 and E, that were blocked by addition of antiestrogens. These observations leaded us to suppose an E2/ERα dependent mechanism for Leydig cell tumor proliferation.

Aromatase expression in rat Leydig cells is driven by the PII promoter regulated mainly by three CRE sites and one SF1 site. Determining the molecular mechanism responsible for aromatase overexpression, we found that total and phosphorylated levels of transcription factors CREB and SF-1 were higher in tumor samples. Moreover, we found that R2C cells produce also high levels of IGF-I that increased aromatase mRNA, protein and activity as a consequence of increased total and phosphorylated SF-1 levels. Binding of IGF-I to its receptor causes receptor autophosphorylation and the activation of an intrinsic tyrosine kinase that acts on various substrates, leading to activation of multiple signaling pathways including the PI3K/AKT and MAPK cascades. In addition, it has been shown that IGF-I

can activate the PLC/PKC pathway (3). Specific inhibitors for IGF-I receptor, Protein Kinase C and Phosphoinositol-3-kinase determined a reduction in SF1 and consequently in aromatase expression and activity. The same inhibitors were also able to inhibit the IGF-1 dependent-SF-1 recruitment to the aromatase PII promoter. These results indicate that in Leydig tumor cells one of mechanism determining aromatase overexpression is an enhanced IGF-I signaling potentiating SF-1 action.

Determining the molecular mechanism responsible for constitutive CREB phosphorylation, we investigate the role of COX-2 (cyclooxygenase-2) an enzyme involved in prostaglandins (PGs) synthesis that has not been detected in the human normal testis, but it is expressed in testicular biopsies of men with cancer. We show that COX-2 is expressed in rat Leydig tumor samples while is not detectable in normal testis. COX-2 specific inhibitor NS398 (5-50 uM) is able to reduce dose-dependently aromatase mRNA, protein expression, activity and tumor Leydig cell proliferation. NS398 significantly decreases CREB activation reproducing the same effect of H89 (a PKA inhibitor) on both aromatase and CREB. The drop in estrogen production determines a decrease in tumor Leydig cell proliferation. Moreover, the addition of increasing amounts of PGE2 were able to increase phosphorylation but not synthesis of CREB which consequently increased aromatase expression. Next, in order to evaluate PGE2 receptor (EP) subtype(s) responsible for induction of aromatase expression and activity, we used the selective inhibitors: SC19220, AH6809, AH23848 for EP1, EP2/EP4 and EP4 respectively. Our data demonstrate that only the AH23848 was able to determine a decrease in CREB phosphorylation and again in aromatase expression. These findings led us to suppose that, in tumor Leydig cells, COX-2-derived PGE2, through an autocrine mechanism, activates PKA which is responsible of CREB activation.

In summary, our results give a contribution to clarify two molecular mechanisms determining aromatase overexpression in Leydig cell tumor. The first one involves some of pathways (PI3-K and PKC) activated the IGF-I determining SF-1 production and enhanced P450 mRNA transcription. The other one involves the production of PGE2 (induced by COX-2 overexpression), which is responsible of PKA activation and CREB

phosphorylation. It remains to elucidate the molecular mechanisms determining IGF-I and COX-2 overexpression in tumoral Leydig cells.

However, the observations that COX-2 and IGF-I pathway inhibitors are able to decrease E2 production and to block Leydig cell tumor proliferation, open new perspectives on trapeutic approach of Leydigioma in the human.

Introduction

1. Endocrinology of the male reproductive system

1.1 The testis: general structure

The human male reproductive system includes the hypothalamic-pituitary-gonadals axis, the epididymis, vas deferens, seminal vesicles, prostate and the urethra. The testis is composed primarily of seminiferous tubules packed closely together and interstitial cells (4). The seminiferous tubules are composed by Sertoli cells that support germ cells during their maturation into spermatozoa. Sertoli cells create a blood-estis barrier, and separate the germinal epithelium into basal and adluminal compartments. They are responsible for the physical support of the germ cells, in addition to providing nutrients and growth factors. The major cell in the interstitial space outside the seminiferous tubule is the Leydig cell, which produces testosterone, a necessary component for germ cell maturation. Male fertility requires the production by the testes of large numbers of normal spermatozoa through a complex process of spermatogenesis. The germ cells are sequentially organised into several layers signifying the respective mitotic or meiotic processes and spermatid development. Each seminiferous tubule is surrounded by mesenchymal cells. Among these are the peritubular myoid cells whose contractile elements generate peristaltic waves along the tubules, but do not present a tight diffusion barrier. Vascular smooth muscle cells, macrophages and endothelial cell types are also located in the interstitial space of the testis. The physiological role of macrophages has long been underestimated. In the rat, the number of macrophages is one quarter of the number of Leydig cells and the presence of macrophages is crucial for (re)population of Leydig cells during development and after experimental depletion (5;6). Immune cells, known to secrete a number of growth factors and cytokines, are part of the intratesticular communication pathways (7).

1.2 Testicular function and its regulation

Testes are components of both the reproductive system (being gonads) and the endocrine system (being endocrine glands). The respective functions of the testicles are:

1. producing sperm (spermatozoa);
2. producing male sex hormones.

These two functions occur in separate compartments within the testis: 1. the seminiferous tubules produce sperm and 2. the interstitial cells (i.e., Leydig cells) synthesize androgens (**Fig. 1**).

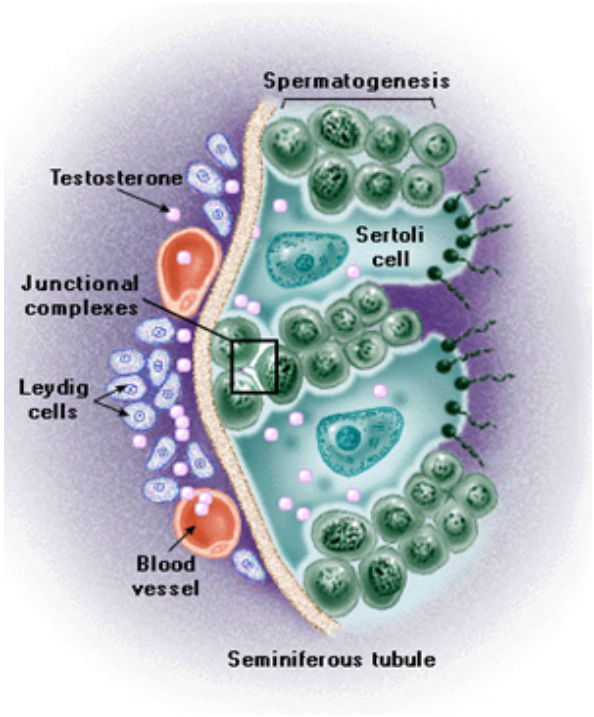


Figure 1. Schematic representation of functions of the testis.

Both functions of the testis, sperm-forming and endocrine, are under control of gonadotropic hormones produced by the anterior pituitary: luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Synthesis and release of both FSH and LH is

regulated by a single gonadotropin releasing hormone (GnRH) also referred to as LHRH, a decapeptide produced by specialized neurons in the hypothalamus. Pulsatile GnRH production signals gonadotroph cells in the anterior pituitary to produce follicle-stimulating hormone (FSH) and luteinizing hormone (LH) that then act on the testis to regulate spermatogenic potential. LH binds to receptors on the surface of Leydig cells in the testis and stimulates the production of testosterone, a steroid hormone that diffuses into the seminiferous tubules. Within the seminiferous tubules only Sertoli cells possess receptors for testosterone and FSH and thus these cells are the major targets of the ultimate hormonal signals that regulate spermatogenesis.

Serum testosterone and inhibin (Sertoli-cell product) downregulate LH and FSH secretion via negative feedback loop. Testosterone also decreases the responsiveness of the pituitary to GnRH (**Fig. 2**).

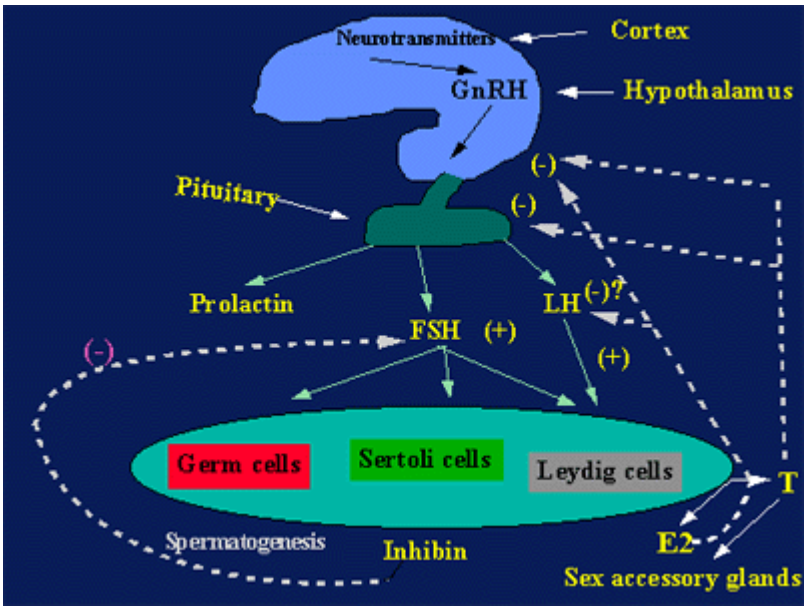


Figure 2. Hypothalamic-Pituitary-Testicular axis.

LH, through specific receptors found on the surface of Leydig cells, controls the production and secretion of testosterone (8;9).

Thus, pituitary gonadotropins are the chief regulators of testicular function. LH stimulates androgen production by Leydig cells after binding to LHR and FSH acts through its receptors in Sertoli cells (FSHR) to regulate spermatogenesis (**Fig. 3**).

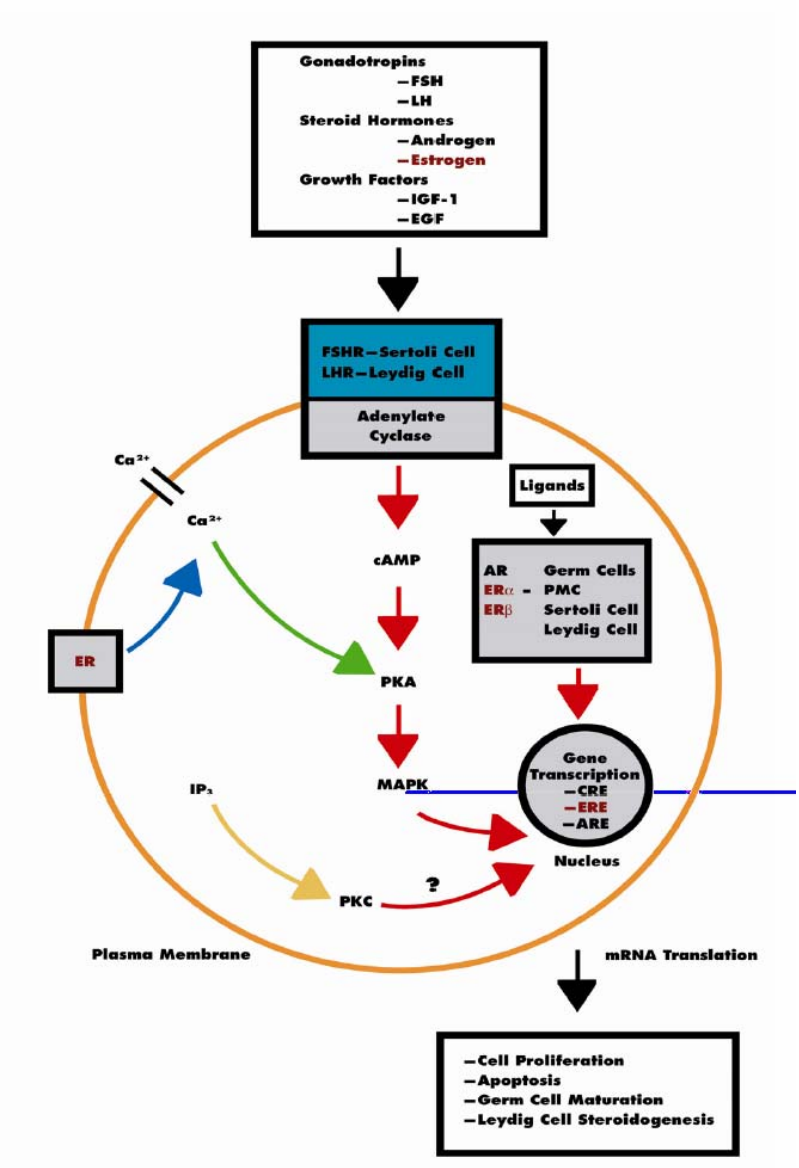


Figure 3. Endocrine regulation of the testis. PMC, peritubular myoid cell; CRE, cAMPresponsive elements, ARE, androgen-responsive elements; ERE, estrogen-responsive elements; (by Akingbemi BT 2005).

The interaction of LH with its receptor initiates signalling through GTP binding proteins determining the cyclic AMP production (10). Signal transduction occurs through the protein kinase A pathway as its principal signal transduction mechanism. Some data suggests that intracellular calcium concentration can be induced by the action of LH by activating phospholipases in the lipoxygenase pathway (11). In addition, the changes in calcium can also regulate adenylate cyclase through the protein kinase C pathway.

Within the seminiferous tubules only Sertoli cells possess receptors for testosterone and FSH and thus these cells are the major targets of the ultimate hormonal signals that regulate spermatogenesis. FSH binding to its receptor is known to activate at least 5 signaling pathways in Sertoli cells: cAMP-PKA pathway, MAP kinase pathway, Phosphatidylinositol 3-kinase (PI3-K) pathway, Calcium pathway, Phospholipase A2 (PLA2) pathway. Initially FSH binding to the FSH receptor causes receptor coupled G proteins to activate adenylate cyclase (AC) and increase intracellular cAMP levels. Multiple factors can be activated by cAMP in Sertoli cells including PKA that can phosphorylate a number of proteins in the cell and also regulate the expression and activity of numerous transcription factors including CREB. During puberty, FSH activates the MAP kinase cascade and ERK kinase in Sertoli cells most likely via cAMP interactions with guanine nucleotide exchange factors (GEFs) and activation of Ras-like G proteins. ERK is capable of activating transcription factors including SRF, c-jun and CREB. In granulosa cells, FSH also activates the p38 MAP kinase. FSH and cAMP also likely act through GEFs to activate PI3-K and then phosphoinositide dependant protein kinase (PDK1) and PKB in Sertoli cells. Studies of granulosa cells identified Forkhead transcription factor (Forkhead), SGK (glucocorticoid-induced kinase) and GSK-3 (glycogen synthase kinase-3) as additional downstream targets of the PI3-K pathway. FSH also mediates the induction of PLA2 and the subsequent release of arachadonic acid (AA) and the activation of eicosanoids such as PGE2 that may act as intracellular or extracellular signaling agents (12). However, gonadal steroids, i.e., androgen and estrogen, and other agents that bind or prevent binding to steroid hormone receptors (androgen receptor AR, ERα, and ERβ), which are present in Sertoli cells, germ cells and Leydig cells also regulate

testicular function (9). The pathway mediated by adenosine-3',5'-cyclic monophosphate (cAMP) appears to be the primary intracellular signaling pathway in all testicular cells. However, several growth factors e.g., insulin like growth factor-1 (IGF-1) and epidermal growth factor (EGF), acting via their receptors, IGF-1R and EGF-R, possibly modulate AR and ER-mediated pathways. Thus, testicular function is regulated by interactions between several signaling pathways, some acting locally, e.g., AR and ER-mediated pathways, and others indirectly by modulating hypothalamus-pituitary function. Hormonal activation of transcriptional gene activity results in changes in cell differentiation and function.

1.3 Steroid production

Testosterone is the major androgen secreted by the testis from its site of production within the Leydig cells. In addition to testosterone, through the actions of the enzyme 5α-reductase, dihydrotestosterone is produced by the testis in smaller amounts. The testis also contributes approximately 25% of the total daily production of 17β-estradiol through the local action of the enzyme aromatase which converts androgenic substrates to this estrogen (13). The remainder of the circulating estradiol is produced by the adrenal and peripheral tissues through the actions of aromatase. Cholesterol represents the major substrate for androgen production by the Leydig cells and is derived by an uptake mechanism involving the binding of circulating low density lipoprotein to specific receptors on Leydig cells which, following internalisation provides a significant source of cholesterol (14). In addition, the Leydig cells are able to undertake de novo synthesis of cholesterol from acetate and relative contributions of these two sources is partly dependent on species and the state of stimulation of the Leydig cells.

The conversion of cholesterol to testosterone involves a number of steps that are catalyzed by enzymes, predominantly belonging to cytochrome P450 family. The mobilization of cellular sources of cholesterol is achieved through the action of cholesterol ester hydrolase and subsequently, this is converted to pregnenolone by the enzyme cholesterol side-chain cleavage termed cytochrome P450SCC (15) (**Fig. 4**).

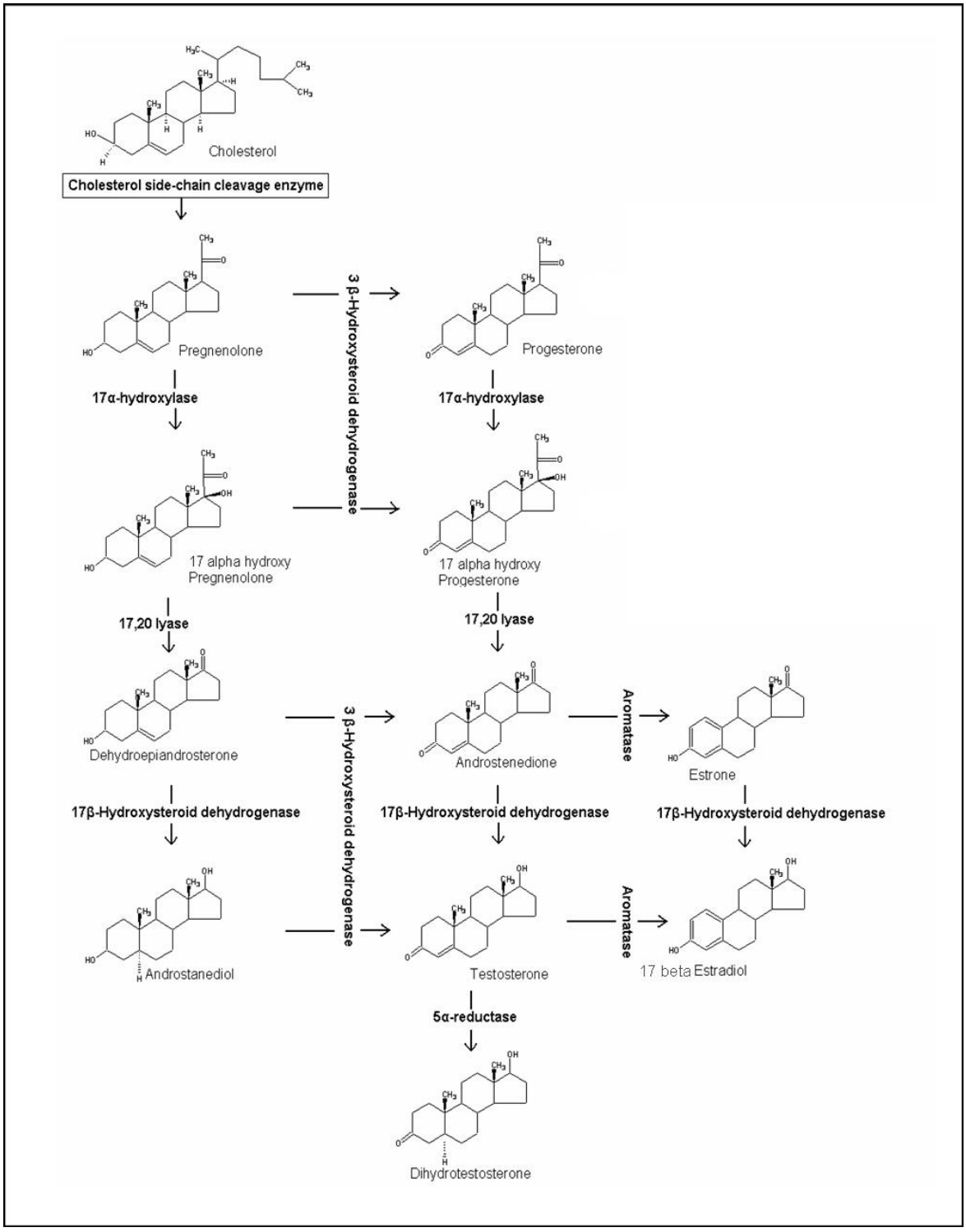


Figure 4. Steps in steroidogenesis leading to androgens and estrogens production.

The conversion of cholesterol to pregnenolone is a key step at which regulation of androgen production within the Leydig cells occurs. Availability of cholesterol substrate can be rate-limiting and the intracellular trafficking of cholesterol across mitochondrial membranes is dependent on the steroidogenic acute regulatory protein (STAR) (16-18).
The role of this protein has been well demonstrated in patients with mutations in the gene encoding STAR in the disorder termed congenital lipoid adrenal hyperplasia wherein the mitochondria from the adrenals and gonads of these patients are unable to convert cholesterol to pregnenolone (19). Further, the results of studies involving targeted disruption of the mouse gene encoding STAR support the data derived from human studies (20). Pregnenolone may progress to testosterone production through two pathways. It can be converted to progesterone through the enzyme 3βhydroxysteroid dehydrogenase (the D4 pathway) or can be hydroxylated at the 17α position by the enzyme 17-alfahydroxylase to form 17α-hydroxypregnenolone (the D5 pathway). The relative importance of these two pathways vary with the species and the physiological status of the male (21). The further conversion of 17α-hydroxypregnenolone through the D5 pathway involves the formation of the C19 steroid dehydroepiandrosterone catalyzed by the enzyme 17,20 lyase and both steps appear to be catalyzed by a single microsomal enzyme cytochrome P450 c17 encoded by a single copy gene on chromosome 10 (22;23). The conversion of dehydroepiandrosterone to androstenediol is mediated by a microsomal enzyme 17β-hydroxysteroid dehydrogenase encoded by a single gene (24;25). The conversion of substrates from the D5 to the D4 pathway are catalyzed by the enzyme 3β-hydroxysteroid dehydrogenase (26). In the D4 pathway 17α-hydroxyprogesterone proceeds through the action of cytochrome P450 c17 to androstenedione and testosterone. Testosterone can be converted to a dihydrotestosterone by the enzyme 5α-reductase (27) or can be metabolised to 17β-estradiol by the enzyme aromatase (13;28).

2. Estrogen regulation of testicular function

Evidence supporting a role for estrogen in male reproductive tract development and function has been collected from rodents and humans. These studies fall into three categories: i) localization of aromatase and the target protein for estrogen (ER-alpha and ER-beta) in tissues of the reproductive tract; ii) analysis of testicular phenotypes in transgenic mice deficient in aromatase, ER-alpha and/ or ER-beta gene; and, iii) investigation of the effects of environmental chemicals on male reproduction. Estrogen is thought to have a regulatory role in the testis because estrogen biosynthesis occurs in testicular cells and the absence of ERs caused adverse effects on spermatogenesis and steroidogenesis (29). All of these topics will be individually discussed in this section of the introduction. In males, estrogens derive from circulating androgens. Aromatization of the C19 androgens, testosterone and androstenedione, to form estradiol and estrone, respectively, is the key step in estrogen biosynthesis, which is under the control of the aromatase enzyme (**Fig. 5**).

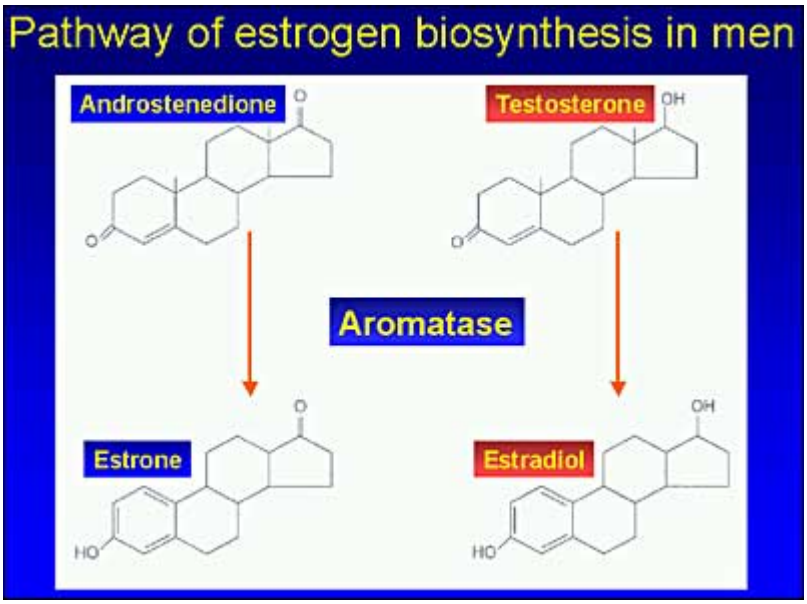


Figure 5. Biochemical pathway of testosterone conversion into estrogens.

2.1 The aromatase gene: structure and regulation

Aromatase is composed of two proteins: a ubiquitous NADPH-cytochrome P450 reductase and a cytochrome P450 aromatase (P450arom), which contains the heme and the steroid-binding pocket. In humans, P450arom is the product of a single gene located in region q21.1 of chromosome 15 and called *cyp19*, which belongs to the cytochrome P450 gene family. The *cyp19* gene is more than 123 kb in length with a coding region of 9 exons (II-X) and 9 nontranslated exons I (30) (**Fig. 6**).

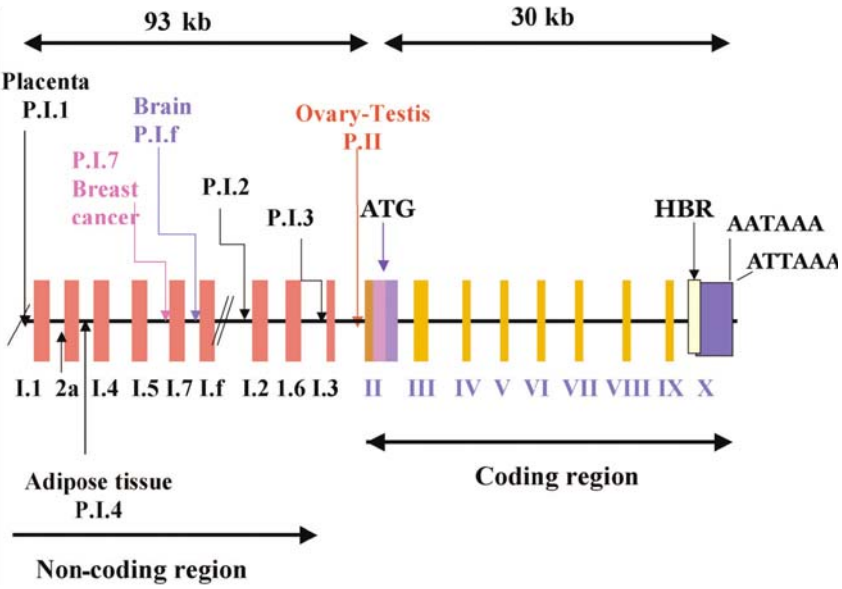


Figure 6. Schematic presentation of the human aromatase gene. P = promoter; (by Carreau S 2007).

Expression of the *cyp19* gene is regulated by tissue-specific promoters producing alternate 5'-untranslated exons I that are then spliced onto a common 3'- splice acceptor site in exon II, upstream of the translation starting site (31-33). Therefore, there is generation of *cyp19* variants with different 5'untranslated regions giving rise to different mRNAs; however, the coding sequences are identical and give rise in humans to a single protein composed of 503 amino acids with a molecular mass of 55 kDa. It is of note that P450arom is encoded by a single *cyp19* gene in most species except for pigs in which three distinct genes encode

three aromatase isoenzymes (34) and for fish in which two *cyp19* genes (specifically expressed in the brain and gonads) have been identified (35). Different mechanisms of regulation of Cyp19 gene expression have been described for various tissues. The synthesis of different aromatase isoforms between species and tissues may involve distinct aromatase genes and/or the function of different promoter elements (36). In human adipose tissue, the primary promoter I.4 lies about 15 kb upstream of the start site of translation (37;38) and is a TATA-less promoter driven by glucocorticoids and class I cytokines e.g. IL-6 and TNFα (33). The region of PII proximal to the translation start site regulates P450arom expression in mammalian gonads (37;39) as well as in Leydig cell tumors (40). Numerous functional motifs have been identified in P.II (33) (**Fig. 7**).

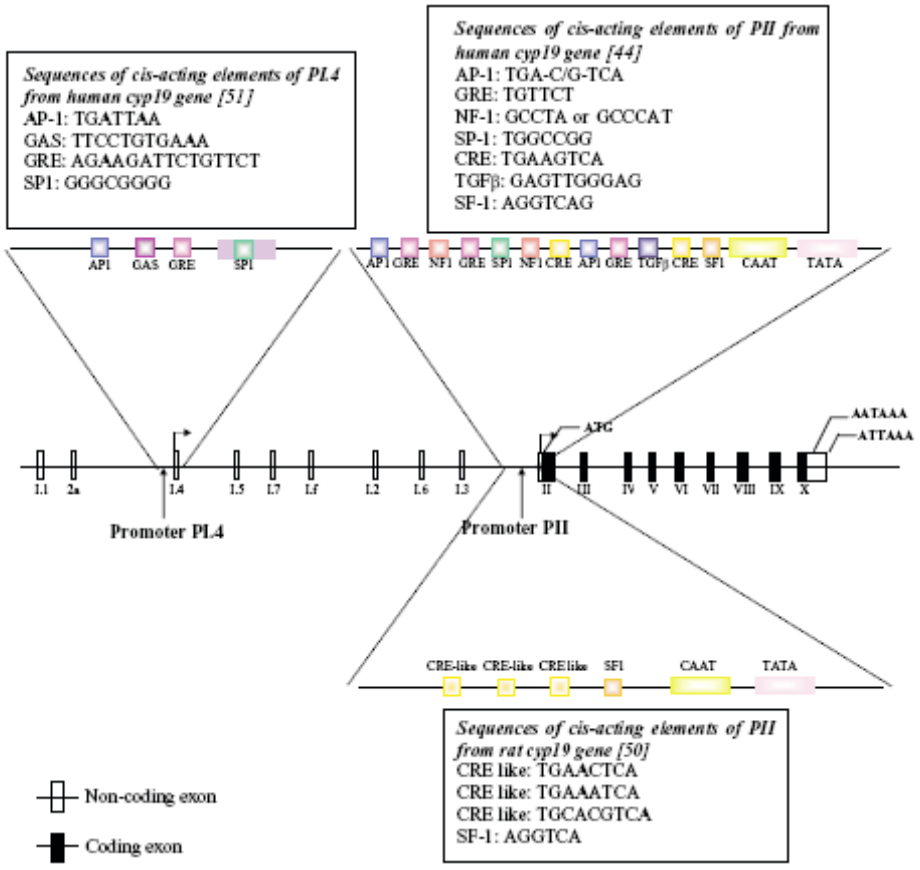


Figure 7. Structure of the human Cyp19 gene showing the various untranslated first exons and their corresponding promoters. The region around promoter PI.4 and PII from human and PII from rat are expanded to show the identified response elements. Sequences of these are shown in boxes; (by Carreau S 2004).

In the testis, FSH and LH act by increasing concentrations of intracellular cyclic AMP to induce expression of P450arom. Promoter PII activity is therefore regulated by cyclic AMP and requires the transcription factors cAMP response element binding protein (CREB), cAMP response element modulator (CREM) and steroidogenic factor-1 (SF-1). SF-1 belongs to the nuclear orphan receptor superfamily and regulates steroidogenic gene transcription (e.g. P450arom via its interaction with numerous coactivators including CREB binding protein, DAX-1, SOX-9, WT1).

It has been shown that the level of P450arom mRNA is increased in Leydig cells of mice deficient for DAX-1 (41). In addition, it is shown that liver receptor homologue-1 (LRH-1), an SF-1 homologue, wich is present in leydig cells and germ cells, but not in sertoli cells, increases the P450arom gene expression in a mouse leydig cell line (42).

Moreover, it is now clear that not only P.II drives the aromatase gene in rat testis but two additional promoters, P.I.f (brain promoter) and a new one that we called P.I.Tr (testis rat;(43), are involved. It is also demonstrated that the nutritional status of fetuses (44) and aging (Hamden K, Silandre D, Delalande C, El Feki A, Carreau S, unpublished results) can modulate aromatase gene expression in male rats.

2.2 The Estrogen Receptors (ERs)

Estrogen actions are mediated by binding to specific nuclear estrogen receptors (ERs), which are ligand-inducible transcription factors regulating the expression of target genes after hormone binding. Two subtypes of ERs have been described: estrogen receptor α (ERα) and the more recently discovered estrogen receptor β (ERβ). The human gene encoding for ERα is located on the long arm of chromosome 6, while the gene encoding for ERβ is located on band q22-24 of chromosome 14.

The two ER (α and β) proteins have a high degree of homology at the amino acid level (**Fig. 8**).

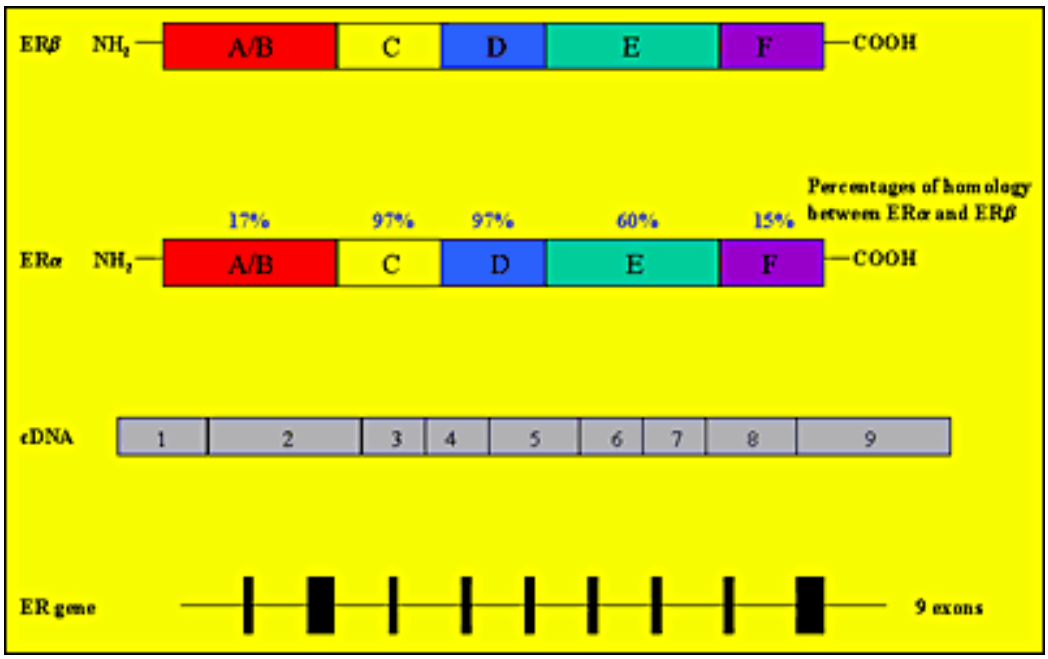


Figure 8. ERs gene and its products (by Akingbemi BT 2005) .

While it is clear that estrogens regulate transcription via a nuclear interaction after binding their receptors, a non-genomic action of estrogens has been recently demonstrated, suggesting a different molecular mechanism accounts for some estrogen actions. In vitro studies showed a very short latency time between the administration of estrogens and the appearance of biological effects. These actions are thought to be mediated through cell-surface receptors, which are not believed to act via a transcriptional mechanism (45). The different types of estrogen action are summarized in Table 1.

Table 1. Estrogen actions and related biomolecular pathways and mechanisms.

Estrogen Actions	Receptors	Mechanism	Final effect	Features
Genomic (nuclear actions)	ERα	Transcriptional: nuclear interaction with estrogen-responsive elements	Modulation of estrogen target gene expression.	Slow effects (minutes or hours)
	ERβ	Transcriptional: nuclear interaction with estrogen-responsive elements	Modulation of estrogen target gene expression.	Slow effects (minutes or hours)
Non Genomic (cell membranes actions)	Estrogen receptors on cells membrane	Cells membrane changes	Changes in ionic transport through cell surface.	Rapid effects (seconds)

ERs are members of the steroid/thyroid hormone super family of nuclear receptors, which share a common structural architecture, and consist of three independent but interacting functional domains: the N-terminal or A/B domains, the C or DNA-binding domain, and the D/E/F or ligand-binding domain (**Fig. 8**). Binding of a ligand to the ER causes a series of downstream events, including receptor dimerization, receptor-DNA interactions mediated by EREs present in the promoter region of target genes, recruitment of and interaction with transcription factors, and the formation of a preinitiation complex.

Ligand- receptor interactions ultimately cause changes in target gene expression (46). The N-terminal domain of nuclear receptors encodes an activation function called AF-1, which mediates protein-protein interactions to induce transcriptional activity. It is thought that this domain is highly active in ERα-mediated stimulation of reporter gene expression from a variety of ERE-constructs but its activity in the ERβ is limited (47). On the other hand, the C-terminal or ligand-binding domain contains the AF-2 interacting surface that mediates ligand binding and receptor dimerization to stimulate transcriptional activity (48).

Thus, AF-1 and AF-2 are both involved in mediating the transcriptional activation functions of ERs. Although there is a high degree of homology in the DNAbinding domains of ERα and ERβ (about 95%), only a partial homology exists in the ligand-binding domain (~60%) (49). Differences in ligand binding, in association with other factors, have the effect of altering the pattern of ER-mediated transcriptional activity. For example, some agonists bind both ER subtypes with the same affinity while others preferentially bind to ERα or ERβ (50-52). There is general agreement that ERs function as dimers, and co-expression of ERα and ERβ in the same cell causes the formation of homodimers (ERα/ERα and ERβ/ERβ) or heterodimers (ERα/ERβ), which affect ligand-specificity. The interactions between ERs and EREs are complicated by other factors, including the ability of ERβ to modulate ERα transcriptional activity and recruitment of several protein co-activators and repressors by both ER subtypes. Therefore, the relative amounts of ERα and ERβ in a given tissue are key determinants of cellular responses to estrogen and other ER agonists and antagonists (53). Moreover, ER and other steroid receptors have the ability to mediate biological effects through non-transcriptional mechanisms mediated by protein-protein interactions occurring between ERs and growth factors e.g., IGF-1 and EGF (54). Furthermore, there is growing evidence for the presence of a small pool of ERs localized to the plasma membrane. For example, BSA-conjugated E2, which is unable to gain entry into the cytosol and acts at the plasma membrane, decreased testicular androgen production *in* vitro (55). Membrane ER is thought to signal mainly by coupling to GTP-activating proteins and through pathways involving second messengers (e.g., calcium) and kinase cascades (56). The integration of several pathways implies that estrogen action in any particular tissue and organ is the result of activities mediated by genomic and non-genomic pathways although the physiological significance of specific pathways in the testis remains to be elucidated (57) .

2.3 Distribution of ERs and aromatase in the male reproductive system

ERs and the aromatase enzyme are widely expressed in the male reproductive tract in both animals and humans, implying that estrogen biosynthesis occurs in the male reproductive

tract and that both locally produced and circulating estrogens may interact with ERs in an intracrine/paracrine and/or endocrine fashion (45). The concept of a key estrogen action in the male reproductive tract is strongly supported by the fact that male reproductive structures are able to produce and respond to estrogens (58).

2.3.1 ERs and aromatase in rodent testis

Aromatase and ERs are found at a very early stage of development in the rodent testis, thus suggesting a role for estrogens in influencing testicular development (59-61). ERα is expressed by Leydig cells in the rodent fetal testis at a developmental stage in which the androgen receptor is not yet expressed. The developing efferent ductules and epididymis also express ERα in the fetal rodent. By contrast, it is unclear whether ERα is present within the seminiferous tubules of the fetal testis, with variable results having been reported (60). ERα is abundant in the developing efferent ductules, which are the first male reproductive structures to express ERs during fetal development (62). ERβ is also found early in testis development in the gonocytes, Sertoli cells and Leydig cells, with the gonocytes showing the highest expression suggesting a role for estrogens in their maturation. In addition, ERβ is expressed by rat Wolffian ducts, the structures from which the efferent ductules and epididymis arise (60). Aromatase is expressed in both Leydig and Sertoli cells in the rodent fetal testis, but not in gonocytes and immature structures of seminal tract. ERs and aromatase distribution in the fetal testes is summarized in Table 2.

Table 2. ERs and Aromatase distribution in the rodent fetal testis.

	ERα	ERβ	Aromatase
Leydig cells	++	++	+
Sertoli cells	-	++	++
Gonocytes	-	+++	-
Seminiferous tubules	+/-	+	+
Ducts	+	+	-

The finding of both aromatase and ERs in the developing fetal testis imply a possible involvement of estrogens in the process of differentiation and maturation of developing rodent testis from an early stage of morphogenesis (59;63). In the postnatal immature rodent testis ERα expression does not occur in the seminiferous epithelium, remaining confined to the Leydig cells, rete testis, efferent ductules and epididymis (Table 3). In the neonatal rodent testis, ERβ is widely expressed by the rat seminiferous epithelium (Sertoli cells and germ cells) as well as by Leydig cells, efferent ductules and epididymis. At this stage ERβ seems to be the only ER in germ cells and is found in pachytene spermatocytes, round spermatids, and perhaps in elongated spermatids of rats and humans (58) (Table 3).

Table 3. ERs and Aromatase distribution in postnatal immature rodent testis.

	ERα	ERβ	Aromatase
Leydig cells	+	+	+
Sertoli cells	-	+	+++
Gonocytes	-	+	-
Seminiferous tubules	-	+	+
Ducts	+	++	(?)

Aromatase is expressed by the dividing Sertoli cells and is stimulated by FSH, with the levels of aromatase declining with age. Fetal Leydig cells also have the ability to produce estrogens in response to LH, but aromatase in this cell type is expressed to a lesser degree than during neonatal life. Interestingly the neonatal testis continues to show a greater degree of aromatase expression in the Sertoli cells than in the Leydig cells (the latter only express aromatase to a greater extent in the adult rat testis when they become one of the major sources of estrogens under the influence of LH) (Table 3). Germ cells in immature rats do not yet express aromatase. ERα is expressed in the Leydig cells of both adult rats and mice (64) but not in Sertoli cells. ERα expression in adult rodent germ cells remains to

be confirmed, with its presence in pachytene spermatocytes and round spermatids being suggested by one study yet its absence demonstrated by others such that the prevailing view is that ERα is absent in germ cells. Studies on the precise cellular localization of ERs expression, however, are mainly based on immunocytochemistry, using different antibodies, and led to contradictory results. Whereas, it is generally agreed that both subtypes are expressed by the epithelial cells of the efferent ductules and epididymis, data concerning testicular expression differ between species, possibly due to different specificity characteristics of the antisera used. Knowledge of the distribution of ERα is of great importance in understanding estrogen action on the male reproductive tract. ERα is highly expressed in the proximal reproductive ducts (rete testis, efferent ductules, proximal epididymis) and its expression progressively decreases distally (corpus and cauda of the epidydimis, vas deferens). The highest degree of ERα expression is seen in the efferent ductules of the rat (65) and accounts for one of the most well-documented estrogenic actions on male reproductive system, that of fluid reabsorption from the efferent ductules. It has to be remarked that the concentration of ERα in the male reproductive tract is opposite to that of ERβ, which is more concentrated in the distal tract (Table 4).

Table 4. ERs and Aromatase distribution in the adult rodent testis.

	ERα	ERβ	Aromatase
Leydig cells	+/-	+/-	+++
Sertoli cells	-	+	+
Germ cells Spermatogonia Pachytene Spermatocytes Round Spermatids Spermatozoa	+/- - (?) -/+ -/+ + (?)	++ + + + + (?)	++++ + (?) + ++ +
Efferent ductules	++++	+	- (?)

ERβ is expressed in Leydig, Sertoli and germ cells in adult rodents (66;67) and has also been detected in primate germ cells (68). There is now considerable evidence that germ cells contain both ERβ and aromatase (68). It should be noted that there are some controversies in terms of ERβ localization, with immunohistochemical studies showing some discrepancies, possibly due to methodological differences. It seems that the regulation of gonocyte multiplication, which is under the influence of growth factors and estradiol, may occur through the involvement of ERβ (69). By adulthood, rodent Leydig cells show higher aromatase activity compared to every other age and in comparison to Sertoli cells (70). Aromatase is also expressed at high levels in germ cells throughout all stages of maturation, and its expression appears to increase as the germ cell becomes a mature spermatid (**Fig. 9**).

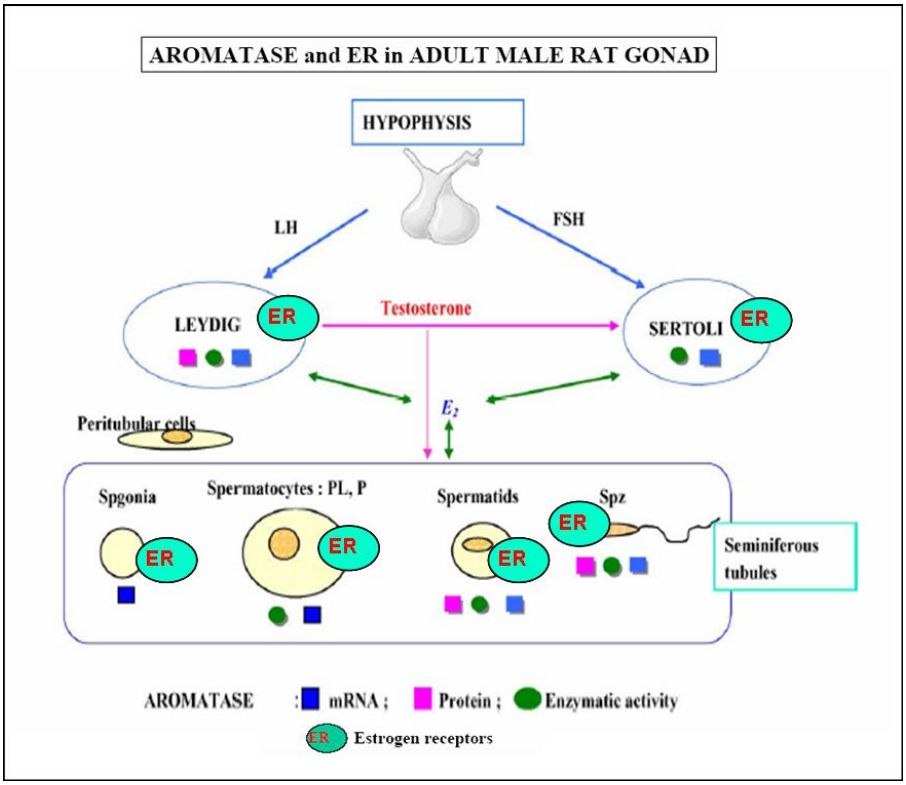


Figure 9. Aromatase and estrogen receptors (ER) in adult male rat gonad. Aromatase has been demonstrated in terms of mRNA (RT-PCR), protein (Western blots) and enzyme activity (measurements of estradiol output in culture media) in the various testicular cells. ER: estrogen receptors localization; (by Carreau S 2003, 2005).

Aromatase mRNA and activity, in fact, are found in germ cells from the pachytene spermatocyte stage in both rats and mice, and during their subsequent maturation into round spermatids(61;70;71). Aromatase seems to be present in higher levels in mature spermatids of the rat than in earlier germ cells (61;71;72). It is of interest that aromatase mRNA expression and enzyme activity is higher in germ cells when compared with Leydig cells, suggesting that germ cells may be a major source of estrogen in adult rodents. When fully developed spermatids are released from the epithelium, aromatase remaining in the residual body is subsequently phagocyted by the Sertoli cell. Some aromatase activity remains in the cytoplasmic droplet that remains attached to the flagellum as the sperm make its way through the epididymis, suggesting that mature spermatozoa are able to synthesize their own estrogen as they traverse the efferent ducts (73;74). The ability to synthesize estrogen gradually decreases as the droplet slowly moves to the end of the tail during epididymal transit until it's finally lost. The demonstration of aromatase in sperm is important as it suggests that the sperm itself could control the levels of estrogen present in the luminal fluid, directly modulating functions such as the reabsorption of fluid from the efferent ductules (65).

2.3.2 ERs and aromatase in the human testis

Both ERs have been found in human testis and reproductive tract. In the male fetus ERβ expression is higher than ERα, the latter being absent or expressed at very low levels. In the human fetus ERβ immunoreactivity has been shown in the seminiferous epithelium (Sertoli cells and a few germ cells) and in the epididymis suggesting a role for ERβ in the prenatal development and function of male reproductive structures (75). In adult men ERα was expressed only in Leydig cells, while ERβ has been documented in both Leydig and Sertoli cells and in the efferent ducts (76). The presence of ERs in the human epididymis is still debated, even though recently ERα has been detected in the nuclei of epithelial cells of the caput of the epididymis (77). Both ERα and β have been detected in human pachytene spermatocytes and round spermatids with in situ hybridization (78-80). These latter studies have been contradicted by more recent studies showing strong expression of ERβ in human

testis but failing to find evidence for ERα using immunohistochemistry (81) and RT PCR (82), suggesting that ERβ is the primary mediator of estrogen action in the human testis. Of particular interest is the demonstration of differential expression of wild type ERβ (ERβ1) and a novel human variant form of ERβ, arising from alternate splicing (ERβcx, or ERβ2), in the human testis (83). ERβ2, which may act as a dominant negative inhibitor of ER action, was highest in spermatogonia and Sertoli cells in adult men, suggesting that these cells may be "protected" from estrogen action by the expression of this variant. However wild type ERβ1 was highest in pachytene spermatocytes and round spermatids, which have been proposed to be estrogen sensitive, yet was low in less mature germ cells (81). As previously suggested by Durkee et al. (84), ERs are present in human sperm. In particular it has recently been documented by Luconi et al. (85) that the sperm membrane contains an estrogen receptor-related protein able to bind steroid hormones that may act through a calcium-calmodulin dependent pathway and thus perhaps accounts for a well documented rapid non-genomic action. Aromatase expression in the human testis is present in both somatic and germ cells from pachytene spermatocytes through elongated spermatids (80;86). Aromatase is also expressed in both human Leydig and Sertoli cells (71). Recently, the presence of aromatase has been demonstrated not only in immature germ cells, but also in mature human spermatozoa (87). In contrast to rodents, aromatase expression in human gametes is not lost during transit through the genital tracts since P450 aromatase was demonstrated in ejaculated human spermatozoa at three different functional levels: mRNA expression, protein and activity (87). Thus ejaculated human spermatozoa continue to express P450 aromatase and contain active aromatase, and thus sperm have to be considered a potential site of estrogen biosynthesis. These evidences support the concept that human spermatozoa should be considered a mobile endocrine unit since they are able to synthesize and to respond to estrogens. Again, the presence of functionally aromatase in human spermatozoa permits the conversion of androgens into estrogens throughout the whole transit of reproductive tract, an event that constantly provides free estrogens in the seminal fluid able to act on the cells of the reproductive ducts.

2.4 Role of estrogens in animal male reproduction

In animals, a previously unsuspected physiological role of estrogens in testicular function was revealed by the creation of the ERα knockout (αERKO) mouse. Adult, sexually mature, male αERKO mice are infertile even though the development of the male reproductive tract is largely unaffected (69). Adult testicular histology shows an atrophic and degenerating seminiferous epithelium, together with dilated tubules and a dilation of the rete testis (88). The disruption of spermatogenesis is progressive as the testicular histology is normal at ten days of age but starts to degenerate at twenty-thirty days. By about 40-60 days the tubules are markedly dilated with a corresponding significant increase in testicular volume while the seminiferous epithelium becomes atrophic. A severe impairment in tubule fluid absorption in the efferent ducts was demonstrated to be the cause of infertility in αERKO male mice, and this defect is partially mimicked also by the administration of an anti-estrogen in wild-type mice (65). In the male genital tract the highest concentration of ERα is found in the efferent ducts (89) and the estrogen-dependent fluid reabsorption in this site probably results from estrogen interaction with the ERα that seems regulate the expression of the Na(+)/H(+) exchanger-3 (NHE3). In fact, the disruption of ERα or the use of antiestrogens result in decreased expression of NHE3 mRNA, as well as in a decrease of other proteins involved in water reabsorption, such as aquaporin I (90;91). The lack of fluid reabsorption in the efferent ductules of αERKO male mice and the consequent dilatation of these ductules induces a retroactive progressive swelling of the seminiferous tubules. The seminiferous tubule damage results from the increased fluid pressure and severely impaired spermatogenesis coupled with testicular atrophy as seen at the age of 150 days (65). In addition, reproductive hormones profiles are abnormal in αERKO male mice as serum LH is significantly increased with a consequent elevated serum testosterone and Leydig cells hyperplasia, but FSH remains in the normal range (69). It is also worth noting that detailed investigations into the development of efferent ductules in αERKO male mice suggest that a congenital absence of ERα leads to developmental abnormalities in this tissue (92). The recent production of both aromatase

knockout (ArKO) (93) and ERβ knockout (βERKO) (94) mice supports the idea that in mice estrogen actions on the male reproductive tract are more complex than previously suggested on the basis of the αERKO mice. In fact, unlike αERKO mice, male ArKO mice are initially fully fertile (93), but fertility decreases with advancing age (95), and, conversely, βERKO mice are fully fertile and apparently reproductively normal in adulthood (94). From seven months of age male ArKO mice are not able to sire any litters. Again histology of the testes of one-year-old ArKO mice shows a disruption of spermatogenesis at the early spermatid without significant (95). The late onset of the alterated phenotype in male ArKO mice is attribuitable to estrogenic substance present in their diet and which are capable of agonistic effect on spermatogenesis (96). Despite the phenotype of αERKO male mice, the mechanism involved in the development of infertility is different in ArKO male mice, since the early arrest of spermatogenesis suggests a failure of germ cell differentiation probably caused by the lack of estrogen action at the level of the seminiferous epithelium rather than a problem referable to impaired fluid reabsorption (59). Recent findings from studies in which human germ cells were treated with estrogen in vitro suggest that estradiol may serve as a survival factor for round spermatids and that lack of estradiol may promote apoptosis with a resulting failure in elongated spermatid differentiation (79). Recently studies in mice deficient in both ER α and β (αβERKO mice) showed a male phenotype very close to that of αERKO mice with infertility and dilated seminiferous tubules (69). These findings, together with the observation that βERKO male mice are fully fertile (94), lead to the hypothesis that estrogen activity in the male reproductive tract differs with regard to both the type of estrogen receptor involved in the pathway of estrogenic action and the site of action through the male reproductive tract. Importantly, results from mice lacking functional ERs or aromatase point to an important role for estrogen in the maintenance of mating behaviour in male mice, and that infertility in αERKO, αβERKO and ArKO mice are at least in part due to reductions in various aspects of mating behavior from an early age. The above studies support the concept that a functional ERα, but not ERβ, is needed for the development and maintenance of a normal fertility in male mice (69). Clearly, further studies are needed to fully understand the

precise role of estrogens and their receptors in the establishment and maintenance of male fertility, and the importance of intracrine and paracrine pathways for these effects.

2.5 Role of estrogens in human male reproduction

The demonstration of abundant ERs in human efferent ducts and aromatase activity in human sperm, speaks in favor of the involvement of estrogens in male reproductive function. On the other hand, data from human subjects with congenital estrogen deficiency have provided conflicting and somewhat confusing results. The only man with estrogen resistance discovered up till now, a human equivalent of the ERKO mouse, had normal testicular volumes and a normal sperm count but with slightly reduced motility (97). The four adult men affected by congenital aromatase deficiency showed a variable degree of impaired spermatogenesis (98-101). The patient described by Carani et al., showed both a severely reduced sperm count and an impairment of sperm viability with germ cell arrest at the level of primary spermatocytes (63). A more recent patient had complete germ cell arrest on testicular biopsy but a semen analysis was not performed according to patient's religious views (98;99). Data concerning the patient described by Morishima et al. are lacking since sperm counts were not analyzed (100). It should be remarked that a clear cause-effect relationship between infertility and aromatase deficiency is not demonstrable in the patient studied by Carani et al., since one of his brothers was infertile despite the absence of mutations in the aromatase gene, suggesting an alternate common cause for their infertility (102). Recently a new patient with aromatase deficiency has been described to have impaired fertility (101), confirming a possible association between congenital estrogen deficiency and infertility. The variable degree of fertility impairment in men with congenital deficiency of estrogen action or synthesis deficiency does not permit a firm conclusion about whether these features are a consequence of a lack of estrogen action or are only epiphenomena, even though a possible role of estrogen on human spermatogenesis is suggested by rodent studies. Recently, the administration of aromatase inhibitors to infertile men with an impaired testosterone to estradiol ratio resulted in an improvement of fertility rate (103), although in the absence of a placebo or control group, these findings

need to be interpreted with great caution. Clearly our knowledge of a role for estrogen in human male reproduction is far from complete. The exposure to the excess of environmental estrogens has been proposed as a possible cause of impaired fertility.

2.6 Effects of excess estrogen on male reproduction

2.6.1 Exposure to excess estrogens in animals

In order to evaluate the effect of estrogen excess on the reproductive tract, several studies have been performed in various animal species treated with diethylstilbestrol, a synthetic estrogenic compound. In male mice, the critical period for Műllerian duct formation is day 13 post-coitus. Prenatal exposure of fetal male mice to DES caused a delay in Műllerian duct formation by approximately two days as well as incomplete Műllerian duct regression with a female-like differentiation of the non-regressed caudal part (104). An increase in the expression of anti-Műllerian-Hormone (AMH) mRNA in male mice fetuses exposed to DES has also been demonstrated. This increase was not accompanied by a regression of the ducts. This data was interpreted to suggest that the asynchrony in the timing of Műllerian duct formation, with respect to the critical period of Műllerian duct regression, led to the persistence of Műllerian duct remnants at birth in male mice. Moreover DES exposure did not impair embryonal genetic development, but increased ERs number, and slightly prolonged the gestation time (cesarean sections were performed to rescue the litter and revealed no difference in size of fetuses from control and DES treated mothers). The timing of DES exposure is crucial to the induction of abnormalities of Műllerian duct development and regression (104). Many studies in rodents suggest that inappropriate exposure to estrogen in utero and during the neonatal period impairs testicular descent, efferent ductule function, the hypothalamic-pituitary-gonadal axis, and testicular function (58). The latter effect can be a direct consequence of exposure to excess estrogen, as well as a secondary effect due to perturbations in circulating hormones or the ability of the efferent ductules to reabsorb fluid. Some studies show that low dose estrogenic substances given during puberty can actually stimulate the onset of spermatogenesis, likely due to

stimulatory effects on FSH (105), highlighting the fact that the effects of excess estrogen on male fertility are often complex. The effects of excess estrogen in the neonatal period can impact upon the testis into adulthood, with permanent changes in testis function and spermatogenesis evident (106).

2.6.2 Aromatase over-expression in rodents

Recently a transgenic line of mice overexpressing aromatase enzyme (AROM+) has been developed (107;108). These mice show highly elevated serum estradiol concentrations, with a reciprocal decrease in testosterone concentrations. The AROM+ males display several of the changes observed in males perinatally exposed to estrogens, such as undescended testes, testicular interstitial cell hyperplasia, hypoandrogenism, and growth inhibition of accessory sex glands. A disruption of spermatogenesis has also been observed which could be a consequence of multiple factors, including cryptorchidism, abnormal Leydig cell function, hypoandrogenemia or hyperestrogenemia. Estrogens are thought to inhibit Leydig cell development, growth and function, resulting in the suppression of androgen production (58). The observation of numerous degenerating germ cells and the absence of spermatids within the seminiferous tubules of AROM+ mice suggest that germ cells development was arrested at the pachytene spermatocyte stage in the cryptorchid testes. Interestingly, the spermatogenic arrest occurred at a stage where P450arom is typically expressed. The spermatogenic arrest found in the AROM+ mice could be explained, at least partially, by the suppression of FSH action. The reduced serum FSH levels in AROM+ males are further evidence of the inhibiting actions of estrogens on FSH secretion in males. No significant differences in the LH concentrations were seen in AROM+ and wild type mice (107;108).

2.6.3 Exposure to excess estrogens in humans

The clinical use of diethylstilbestrol (DES) by pregnant women in order to prevent miscarriage resulted in an increased incidence of genital malformations in their sons (109). In these individuals the presence of Műllerian ducts remnants was found indicating that fetal exposure to DES may have an effect on sex differentiation in men, as is the case in rodents (104). Moreover a large number of structural and functional abnormalities were found, the most frequent being: epididymal cysts, meatal stenosis, hypospadias, cryptorchidism and microphallus (109). The frequency of abnormalities was dependent on the timing of estrogen exposure: in fact, men who were exposed to DES before 11th week of gestation (i.e. the time of Műllerian ducts formation) had a two fold higher rate of abnormalities than those who were exposed only later (109). This data supports the previously discussed hypothesis that the asynchrony between formation and regression of embryonal reproductive structures is determined by estrogen exposure. Various reports have demonstrated that semen quality of men exposed to DES in utero is significantly worse than in unexposed controls (110;111). However, the sperm concentrations of most of the DES exposed men were well above the limit at which subfertility occurs, and it is therefore not surprising that the fertility of these men was reported to be normal (112). The risk of testicular cancer among men exposed to DES in utero has been a controversial issue and several meta-analyses showed no increased risk (113). However more direct evidence will be necessary in order to fully understand this issue. While various studies suggest that environmental estrogens affect male fertility in animal models, the implications for human spermatogenesis are less clear (114). It has been demonstrated that male mice whose mothers have consumed a 29 ng/g dose of bisphenol A for seven days during pregnancy had a 20% lower sperm production as compared to control males (115). Various abnormalities in reproductive organs have also been described in males exposed to bisphenols (i.e. a significant decrease in the size of the epididymis and seminal vesicles and an increase in prostate gland volume), suggesting that bisphenols interfere with the normal development of the Wolffian ducts in a dose-related fashion. Exogenous estrogens

could interfere with the development of the genital structures if administered during early organogenesis, by leading to both an impairment of gonadotropin secretion and by creating an imbalance in the androgen to estrogen ratio, which may account for impaired androgen receptor stimulation or inhibition according to the dose, the cell type and age (1;116;117). An excess of environmental estrogens has been suggested as a possible cause of impaired fertility in humans (118). A progressive decline in sperm count has been reported in some Western countries during the past 50 years, suggesting a possible negative effect of environmental contaminants on male reproductive function (119). Data concerning the role of estrogens in male reproductive structure development remains conflicting. Animal studies suggest that exposure to estrogen excess may negatively affect the development of reproductive male organs. These effects, however, are considered to be the result of an impaired hypothalamic-pituitary function as a consequence of estrogen excess and of the concomitant androgen deficiency (1;117). Much of the knowledge on excess estrogen exposure and human fertility depends upon animal data and the validity of these concepts to humans has not been established.

2.6.4 Aromatase over-expression in humans

In 1996 a boy with aromatase excess syndrome was reported (120). His condition was presumably inherited in an autosomal dominant fashion with sex-limited expression as his father had a history of peripubertal gynecomastia, elevated serum estrogen levels and increased aromatase activity in vitro. The father was fertile and had a normal libido despite a small testicular volume (15 mL bilaterally), and a reduced testosterone level of 234 ng/dL (120). In the son, mild suppression of testicular growth and Leydig cell function probably reflected direct estrogen negative feedback on pituitary gonadotropin secretion. In general, the inhibitory effects of estrogen on reproductive function appear to be milder in males with aromatase excess syndrome than in patients receiving exogenous estrogens or with estrogen-secreting tumors, probably because serum estradiol and/or estrone levels are lower in the former (120).

3. Testicular cancer

3.1 Introduction

Although cancer of the testes is rare, accounting for only about 1 percent of all cancers in men of all ages and about 5 percent of all male genitourinary system cancers, it is the most common cancer in men between the ages of 15 and 35, and the second most common malignancy in men ages 35 to 39 (121-124).

Because the incidence of testicular cancer has risen markedly in the past 20 years, numerous studies are being conducted to explore possible environmental causes, including the mother's diet during her pregnancy as well as her use of diethlstilbestrol (DES) to prevent miscarriage. Researchers are also looking at the increasing presence of estrogen-mimicking pollutants in the environment. The most consistent occupational association has been the elevated rate among men in professional and white-collar occupation, which may be linked to an increased risk observed with lower levels of exercise. Other possible causes include hereditary factors, genetic anomalies, congenital defects involving the reproductive tract, testicular injury, and atrophy of the testes. Viral infections such as mumps, which cause inflammation of the testes, have not been proven to cause cancer.

Testicular cancer comprises a number of different diseases. Nearly all of the main cell types in the testis can undergo neoplastic transformation, but germ cell-derived tumors constitute the vast majority of cases of testicular neoplasms. Ninety-five percent of testicular cancers arise from sperm-forming, or germ cells and are called germinal tumors. The remaining 5 percent are nongerminal tumors. About 40 percent of germinal tumors are categorized as seminomas. Several other types of germinal tumors are referred to collectively as non-seminomas. Somatic cell tumors, known as sex cord-stromal neoplasms and Leydig cell tumors are relatively rare. However, being derived from endocrine active cells, they have endocrine manifestations.

3.2 Leydig cell hyperplasia and tumors

Although Leydig cells in adult men are considered to be a terminally differentiated and mitotically quiescent cell type, in various disorders of testicular function, focal or diffuse Leydig cell hyperplasia is very common. Micronodules of Leydig cells are frequently seen in certain conditions associated with severe decrease of spermatogenesis or germinal aplasia, such as the so-called Sertoli-cell-only syndrome (Del Castillo syndrome), cryptorchidism, or Klinefelter's syndrome (125). A term "Leydig cell adenoma" is used when the size of a nodule exceeds several fold the diameter of a seminiferous tubule. It is unknown whether Leydig cell adenomas can progress further to form overt Leydig cell tumors, but even if it was the case, it is exceedingly rare. Morphological heterogeneity of hyperplastic Leydig cells is noticeable in some cases.

The mechanism of Leydig cell hyperplasia in the human male is still poorly understood. The disruption of hypothalamo-pituitary-testicular axis leading to an excessive stimulation of Leydig cells by LH can play a central role (125). However, molecular pathways remain largely unknown in the vast majority of cases. In a small subset of cases structural changes of the LH receptor (126;127) and G proteins (128;129) were detected. Constitutively activating mutations of LH receptor cause early Leydig cell hyperplasia and precocious puberty (126;130). Similarly, constitutively activating mutations of Gs-protein in Leydig cells lead into hyperplasia and endocrine hyperactivity (129;131). However, Leydig cell hyperplasia is distinct from tumors that are usually solitary, and the role of the LH receptor and G protein mutations in the tumorigenesis may be limited to few cases (127;129). Leydig cell hyperplasia and adenomas can be easily induced in rodents by administration of estrogens, gonadotropins and a wide range of chemical compounds. Whether or not humans would be similarly susceptible to environmental effects remains to be elucidated.

Leydig cell tumors account for one to three percent of testicular neoplasms and occur in all age groups (131-133). Approximately 20 % are found before the age of 10, most often between five and ten years of age. Precocious puberty is the presenting symptom in these cases. Tumors produce androgens, mainly testosterone in a gonadotropin independent

manner, and therefore LH and FSH remain low in spite of external signs of puberty. Approximately 10 % of the boys also have gynecomastia that is caused by estrogens produced in excess due to aromatase activity. In adults, gynecomastia is found in approximately 30 % of patients (133). The excessive androgen secretion rarely causes notable effects in adults.

Leydig cell tumors are always benign in children and can be treated with surgical enucleation when the tumor is encapsulated (123), whereas in adults malignant tumors have been found in 10-15 % of patients, and inguinal orchidectomy remains the treatment of choice (132). The presence of cytologic atypia, necrosis, angiolymphatic invasion, increased mitotic activity, atypical mitotic figures, infiltrative margins, extension beyond testicular parenchyma, and DNA aneuploidy are associated with metastatic behavior in Leydig cell tumors (133;134). Malignant tumors are hormonally active only in exceptional cases. Benign tumors can be treated by orchidectomy, whereas an additional retroperitoneal lymphadenectomy should be considered when the gross or histological features suggest malignancy (134). Malignant tumors have not responded favorably to conventional chemotherapy and irradiation (134). Survival time has ranged from 2 months to 17 years (median, 2 years), and metastases have been detected as late as nine years after the diagnosis (133;134). Therefore follow-up of patients with malignant Leydig cell tumors has to be life-long. The remaining testis may be irreversibly damaged by longstanding high estrogen levels, resulting in both permanent infertility and hypoandrogenism(133-135).

The most frequently encountered testicular neoplasm of the mouse and rat is the Leydig cell adenoma. Incidence rates vary in different strains with the Sprague-Dawley SD rat ranging from 1 to 5% and the F-344 rat reaching nearly, 100% (136). Early neoplasm are common in 1 yr old F-344 rats and become increasingly more frequent with age (137). Testicular neoplasia is less frequently observed in alla strains of mice with incidence ranging from 1 to 2,5%. Leydig cell tumors in rodents generally occur in older animals, but in human can arise in any age, the majority between 20 and 60 yr (138). The estimated incidence in man is 0.1-3 per million. The proliferative lesions in Leydig cells in rodents are similar and are observed as a continuous spectrum starting with smaller nodular foci of

hyperplasia leading to large Leydig cell adenomas that can eventually replace the entire testis. The distinction between hyperplasia and adenoma is not always clear, with size being the major factor in the diagnostic criteria, with some debate over when focal hyperplasia becomes early neoplasia and there can be little morphological difference between a hyperplastic nodule and a small Leidig cell adenoma. The major difference between the testicular tumors observed in human and rodents (particularly the rat) are the high incidence of germ cell tumors in human and their occurrence in relatively young men. In rats, germ cell tumors are extremely rare, but Leydig cell tumors can be almost 100% in incidence in certain strains (e.g., Fisher F-344) and occur most frequently in older animals.

3.3 Relationship between estrogens and Leydig tumors

The biological significance of estrogen-induced testicular tumorigenesis has been suggested by the in vivo model overexpressing aromatase transgenic mice (139). Half of these males were infertile and some of them showed larger than normal testis and Leydig cell hyperplasia/Leydig cell tumor. Furthermore, aromatase was markedly immunolocalized in the cytoplasm of interstitial cells, and its immunoreactivity appeared to be strongest in the testes with more advanced stages of neoplasia. The same transgenic animals exhibited estrogen circulating levels at least twice higher than those of control animals and the levels of aromatase mRNA in their testicular tissues were fourfold higher when compared with controls. It is worth to mention how ERα protein in testicular tissue of aromatase transgenic animals was very high with respect to the undetectable levels of control animals. So the authors suggest how an enhanced synthesis of estrogens in tumoral tissues led to an upregulation of ERα expression. Human Leydig cell tumor is a rare testicular neoplasm where estrogen involvement in tumorigenesis process has scarcely been investigated. Recently, a strong aromatase expression in tumoral tissues was revealed by immunostaining and western blotting (140). This finding agrees with a single previous report (141) showing the aromatase immunolocalization in Leydig cell tumors. Furthermore, aromatase expression in control human testicular tissue confirmed Turner's

report in normal testes (142). The enhanced endogenous synthesis of estrogens by Leydig cell tumor was reflected in both patients by a dramatic increase of estrogen circulating levels, resulting more than twofold higher than those of adult normal male, and by the low testosterone levels (at the lower limit of normal range) (140). Moreover, the ratio between the free fraction levels of the two steroids is furthermore increased in the target tissues. The diminished sperm count and motility of both patients may not only be related to altered testicular tropism, parenchymal compression, and increased local temperature ipsilateral to the tumor (143) but also to the detrimental effects of high circulating estrogen levels on the counter-lateral gonad activity. In the adult normal male, 80% of the plasma estradiol originates from aromatization of testosterone and androstenedione in fat, striated muscle, and other tissues including bone and brain, while 20% in the circulation is secreted by the testis. So, it is reasonable to argue how the excessive increase of estradiol circulating levels, observed in the two patients with Leydig cell tumor, is the consequence of an enhanced rate of testicular secretion. This is confirmed by the evidence that estradiol, as well as E2/T ratio circulating levels, drops dramatically following surgical treatment, while for one of the two patients the persistence of a conspicuous bilateral gynecomastia led to bilateral mastectomia (140). Following orchidectomy, the two patients exhibited a moderate increase of sperm count and a remarkable augment of sperm motility (140). The latter event may be reconducted to the restored testosterone circulating levels likely affecting the entire male genital tract. The expression of ER isoforms in Leydig cell tumor is, to date, unknown. In fact, only a single work showed the ER immunolocalization in cryostat sections of Leydig cell tumor (24); recently, immunohistochemical and western blot analysis of tumoral tissues revealed the expression of ERα and of the two ERβ isoforms, ERβ1 and ERβ2, in neoplastic Leydig cells of both patients. So, the pattern of ERs expression in tumoral cells appears different from that of control Leydig cells, exhibiting only ERβ1 and ERβ2 as previously reported (81;83) .

There is a growing body of evidence that ERα and ERβ can be expressed together in the same cell type and independently expressed in another. Therefore, homodimers (ERα–ERα/ERβ–ERβ) or heterodimers (ERα–ERβ) can be formed (28). The binding affinity of

ERα–ERα/ ERα–ERβ dimers for a consensus DNA estrogen response element is reported to be higher than that of the ERβ –ERβ homodimer (29). Thus, the presence of ERα could reinforce the estradiol-induced tumor cell proliferation. Finally, has been demonstrated that neoplastic Leydig cells are potential estrogen biosynthesis sites and display a modified ER expression pattern. Therefore, it appears reasonable to suggest that the high estrogen levels, measured in the two patients, could play a role in the neoplastic transformation of Leydig cells, while the exclusive presence of ERα in tumoral cells could amplify E2 signaling contributing to the tumor cell growth and progression.

4. The IGF system

4.1 Introduction

The insulin-like growth factor (IGF) signalling axis involves the coordinated function of two ligands, IGF-I and IGF-II, three cell surface receptors, at least six high affinity binding proteins and binding protein proteases). This signalling axis plays a pivotal role in normal growth and development (144), and is also implicated in mediating many aspects of the malignant phenotype in a variety of human malignancies (145-147).

IGF-I and -II are growth-promoting peptides, members of a superfamily of related insulin-like hormones that includes insulin and relaxin in the vertebrates and bombyxn, locusta insulin-related peptide, and molluscan insulin-like peptide in invertebrates (148-153). However, insulin and IGFsare the most closely related in terms of primary sequence and biological activity. The IGFs are major growth factors, whereas insulin predominantly regulates glucose uptake and cellular metabolism. Them both are secreted as prohormones and undergo trough a proteolitic process to produce the active peptide.

Structurally, they consist of A, B, C, and D domains (**Fig. 10**).

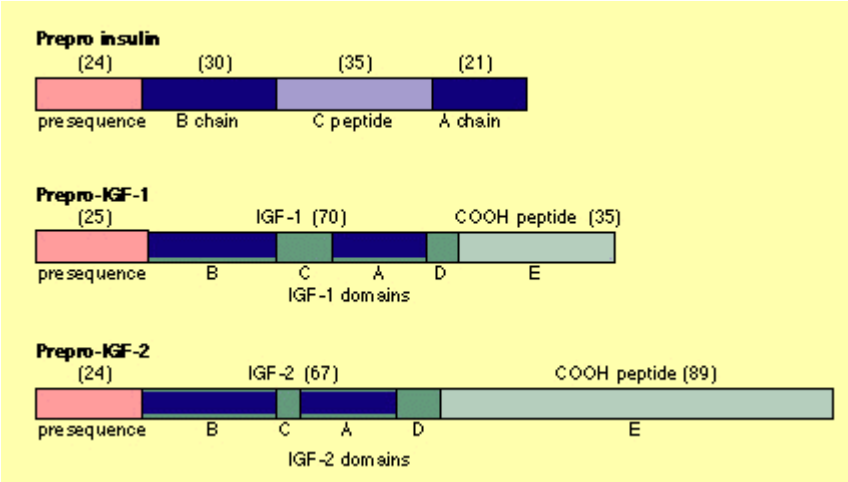


Figure 10. Protein domains in IGFs and Insulin. Regions showing homologies with insulin are indicated by dark blue rectangles. The lengths of individual fragments (in amino acids) are indicated in brackets.

Large parts of the sequences within the A and B domains are homologous to the α- and β-chain of the human proinsulin. This sequence homology is 43% for IGF-I and 41% for IGF-II. No sequence homology exists between the C domains of IGFs and the C peptide region of human proinsulin. The gene encoding IGF-I is highly conserved (154) such that 57 of 70 residues of the mature protein are identical among mammals, birds, and amphibians (155-157). Expression of the IGF-I gene is affected at many levels including gene transcription, splicing, translation, and secretion. IGF-I expression is also influenced by hormonal (GH) (158), nutritional (159), tissue-specific and developmental factors (160), The biological actions of the IGFs are mediated by the type I IGF receptor (IGF-IR), a glycoprotein on the cell surface that transmits IGF binding to a highly integrated intracellular signaling system (161). Binding of IGF-I to its receptor causes receptor autophosphorylation and the activation of an intrinsic tyrosine kinase that acts on various substrates, leading to activation of multiple signaling pathways including the PI3K/AKT and MAPK cascades. In addition, it has been shown that IGF-I can activate the PLC/PKC pathway (3). IGF-II also binds to the IGF-IR buth with lower affinity (161). Expression of the IGF-IR gene (162) has been detected in many tissues and is constitutively expressed in most cells (163;164); its promoter is regulated in vitro and in vivo by transcription factors such as Sp1 and the transcription factor p53 (165). Various IGF-I receptor subtypes that present distinct structures or binding properties have also been described. Two of these subtypes, namely hybrid and atypical IGF-I receptors, have been particularly investigated in a variety of cell types (166). The atypical IGF receptors are characterized by their ability to bind insulin as well as IGFs with relatively high affinity (167). Hybrid insulin/IGF-I receptors have been reported in cells expressing both IGF-I and insulin receptors (168); however, the physiological significance of hybrid and atypical IGF receptors is unclear. The IGF-II ligand has greatest affinity for a distinct receptor, the type-II or IGF-II receptor (169). This single chain polypeptide with a short cytoplasmic domain lacking tyrosine kinase activity is identical to the cation-independent mannose-6 phosphate (M6P) receptor (170). The IGF-II/M6P receptor binds two general classes of ligands: 1) non-M6P-containing ligands, the best characterized of which is IGF-II; and 2) M6P-containing

ligands, including lysosomal enzymes. The multifunctional role of the receptor is evidenced by its function in the mediation of lysosomal enzyme trafficking, endocytosis, and lysosomal degradation of extracellular ligands, regulation of apoptotic/mitogenic effects, and possible intracellular signal transduction (171;172). More recently, high-affinity binding of IGF-II to an insulin receptor isoform (IR-A) has also been reported (173), thus suggesting that IGF-II might also signal via the insulin receptor. However, this insulin receptor isoform (IR-A), lacking the alternative spliced exon 11, is preferentially expressed in fetal and cancer cells (174).

A family of six high-affinity IGF-binding proteins (IGFBP-1 through IGFBP-6) coordinate and regulate the biological activity of IGF in several ways: 1) transport IGF in plasma and control its diffusion and efflux from the vascular space; 2) increase the half-life and regulate clearance of the IGFs; 3) provide specific binding sites for the IGFs in the extracellular and pericellular space; and 4) modulate, inhibit, or facilitate interaction of IGFs with their receptors (175;176). IGFBP biological activity is regulated by posttranslational modifications such as glycosylation and phosphorylation and/or differential localization of the IGFBPs in the pericellular and extracellular space (175;176). It is therefore hypothesized that IGFBPs, in addition to stabilizing and regulating levels of diffusible IGFs, might regulate IGF-I cellular responses by facilitating receptor targeting of IGF-I or modulating IGF-I bioavailability in the pericellular space. The effects of the IGFBPs are further regulated by the presence of specific IGFBP proteases, which cleave the binding proteins, generating fragments with reduced or no binding affinity for the IGFs (177). Some IGFBPs, including IGFBP-2 and -3, can induce direct cellular effects independent of the IGFs (176;178). IGFBP-3, similar to IGFBP-5, contains sequences with the potential for nuclear localization (179) and detection of IGFBP-3 in the nuclei of dividing cells, as reported by several investigators (180), strongly suggesting a role for IGFBP-3 in gene regulation. More recently, perinuclear or nuclear localization has also been reported for IGFBP-2 (181); however, the role of IGFBP-2 in this cellular compartment is yet to be determined.

4.2 Effect of Insulin-Like Growth Factor I on testicular function

Although it is well established that testicular function is mainly controlled by the gonadotropins LH and FSH, there is now considerable evidence indicating that local factors are extremely important in regulating the functions of the testis (182). For example, higher insulin levels in testicular fluid have suggested a role for this factor in Leydig cell development and function (183). Another factor, insulin-like growth factor I (IGF-I), is believed to be a potent para/autocrine stimulator of Leydig cell function (184). Several laboratories have demonstrated IGF-I immunoreactivity (185;186) and IGF-I messenger RNA (mRNA) (187) in the adult rat testis. Immunostainable IGF-I has been found in adult human testes (188). Cultures of Sertoli and Leydig cells from adult rats and immature pigs secrete immunoreactive IGF-I into the medium, and this secretion is enhanced by FSH (Sertoli cells) or LH (Leydig cells) (189;190). Type I receptors for IGF-I have been found on human, pig, and rat Leydig cells (191;192), and IGF-I enhances the differentiated functions of Leydig cells (193). IGF-I stimulates the hCG-supported production of cAMP and testosterone by cultures of rat (194) and pig (195) Leydig cells. The response to cAMP analogs is also enhanced (195), suggesting that IGF-I potentiates the action of LH/hCG at sites both proximal and distal to cAMP generation. IGF-I increases the number of LH/hCG receptors (192) and the amount of LH/hCG receptor mRNA (196) as well as the activities of several steroidogenic enzymes and the amounts of mRNAs encoding them (197-199). The role of IGF-I has been demonstrated in testicular growth and development, control of Leydig cell numbers, and in the onset of steroidogenesis and spermatogenesis (184;200). Lastly, the crucial role of IGF-I in the development and function of Leydig cells was obtained in studies of IGF-I gene knock-out mice(201). The testes of these animals were reduced in size and had fewer and smaller Leydig cells than normal, and the plasma testosterone levels were markedly reduced. It has recently been demonstrated that IGF-I null mice have decreased levels of serum testosterone and steroidogenic acute regulatory (StAR) protein (202). StAR has been demonstrated to play an essential role in regulating steroid biosynthesis by mediating the transfer of cholesterol from the outer to the inner

mitochondrial membrane where it is converted to pregnenolone (203). Transcriptional and/or translational inhibition of StAR expression results in a dramatic decrease in steroid biosynthesis whereas approximately 10–15% of steroid synthesis appears to be mediated through StAR-independent mechanisms (204). Recent findings indicate that IGF-I is capable of increasing expression of StAR protein and steroid synthesis in mouse Leydig cells (3).

4.3 IGF system and tumorigenesis

IGF-I is a peptide hormone that is involved in controlling proliferation and differentiation. Although most of the IGF present in circulation is protein bound, a small fraction of IGF-I is "free"; this component may be more bioavailable, but assays specific for free ligand are controversial. The IGF binding proteins IGFBP-3 and IGFBP-1 both affect IGF-I bioavailability and, in addition, seem to exert independent effects on the growth control of malignant cells (205) as part of a comprehensive regulation system of cell survival and death. Several reports indicate that high circulating levels of IGF-1 are associated with increased risk of developing breast, colorecatal, prostate and skin cancer. In fact, a positive association between circulating levels of IGF-I generally and breast cancer risk was observed in premenopausal women (206). However, results from observational studies have not been consistent (206;207) and considerable uncertainty remains regarding the true association between IGF-I and premenopausal breast cancer risk. Furthermore, the association between IGFBP-3 and breast cancer risk is also inconsistent. In part, these inconsistencies may be attributed to technical variation in performance of assays for IGFs, particularly IGFBP-3, the primary IGF-I binding protein. Other potential explanations for inconsistencies in results include differing blood sampling and storage methods, different definitions of cancer "cases," differences in age at blood sampling, and the possibility of differences between populations in factors that may influence the IGF-I risk relation.
Several studies suggest that IGF-I and IGF-II are important in the pathophysiology of colorectal carcinoma. IGF receptors are found in human colon cancers (208), and full-

length messenger RNAs for IGFs have been detected in human tumor cells (209). Exogenous IGF-I and -II stimulate proliferation of human colorectal cancer cells (210), whereas blockade of the IGF-I receptor inhibits tumor cell growth (211). Individuals with acromegaly, a disease of somatic growth caused by increased growth hormone and IGF-I, have an increased incidence of colonic cancer (212). IGF-binding protein-3 (IGFBP-3) binds more than 95% of the IGF in serum and influences cell proliferation by modulating access of IGFs to the IGF receptors (213). IGFBP-3 also apparently inhibits growth and induces apoptosis through IGF-independent mechanisms (214). Most circulating IGF-I and IGFBP-3 are synthesized in the liver, where expression of each is increased by growth hormone. There is considerable between-person variability in blood levels of IGF-I, IGF-II, and IGFBP-3 (215). Tissue IGF bioactivity is influenced by circulating IGF levels and by local expression of IGFs, IGFBPs, and IGFBP proteases (175). Some factors that regulate determinats of local IGF bioactivity may regulate circulating IGF-I levels in a parallel fashion (216).

5. The cyclooxygenase enzymes (COX)

5.1 Structure, function and regulation of COX

The prostaglandin endoperoxide H synthase (also COX) is a rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandin H2, the precursor of various molecules including prostaglandins, prostacyclin, and thromboxanes (**Fig 11**).

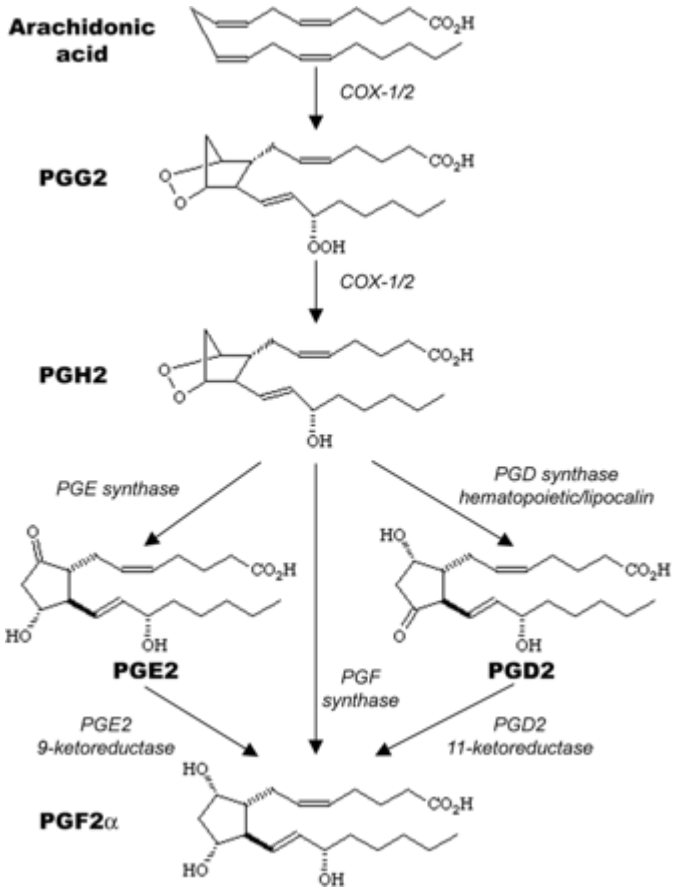


Figure 11. Schematic representation of the COX pathway illustrating the synthesis of the major PGs. This process is initiated when COX-1 and COX-2 catalyze both a reaction in which arachidonic acid is converted to PGG2 and a subsequent peroxidase reaction in which PGG2 is reduced to PGH2, which serves as the common precursor for all of the terminal PGs.

Two COX genes, *COX-1* and *COX-2*, which share over 60% identity at the amino acid level have been identified (217). The gene contains 10 exons is 7.5 kb in length and located at chromosome 1. The transcriptional start site was mapped at 134 bases upstream from the ATG start codon. Nucleotide sequence of 1.8 kb promoter region contains a TATA box and a number of potential regulatory elements including CRE, NF-kappa B, Sp1 and AP2 sites. The linear sequence and three-dimensional structure of the two isoforms are very similar. Even the active site of the isoforms differs minimally (valine/isoluecine substitutions) at only two positions. Nevertheless, inhibitors with high and clinically demonstrable selectivity for the isoforms are available (218-220). COX-1 is constitutively expressed in most cells, thus regarded as a housekeeping molecule, and is responsible for various physiological functions including cytoprotection of the stomach, vasodilatation in the kidney, and production of a proaggregatory prostanoid, thromboxane, by the platelets. On the other hand, the expression of COX-2 is inducible and remains undetectable in most mammalian tissues under basal conditions. Exposure of several types of cells including fibroblasts, endothelial cells and monocytes to bacterial endotoxins, cytokines, hormones or growth factors induces its expression within 2–6 h. Historically, gram (−) bacterial LPS was the first inducer of COX-2 expression to be identified in macrophages (221). It is now known that most pro-inflammatory mediators induce the expression of COX-2. More specifically, LPS and other TLR ligands bind to MyD88-associated receptors and via MEK/ERK induce the transcription factor activator protein 1 (AP1). LPS also activates the TRAF6/NIK/Tpl2/IKK/NFkB pathway, which also leads to induction of COX-2 transcription. Tpl2 signals also lead to ERK1/2 activation, which in turn activates p90RSK and MSK1, which phosphorylate CREB, a central regulator of COX-2 transcription (222). LPS activates C/EBPβ and C/EBPδ via p38MAPK and ERK1/2. It should be noted that C/EBPβ and CREB play a major role during the initial stage of COX-2 transcriptional activation while C/EBPδ maintains an already induced transcription (223). The transcription complex at the COX-2 promoter requires the transcriptional co-activator p300. It now appears that p300 binds on CREB, AP1, C/EBP and NFkB, controlling the initiation of transcription (224). Nitric oxide (NO) affects COX-2 directly by increasing its

catalytic activity and indirectly by triggering several signalling cascades that affect transcription. Thus, NO reacts acutely with superoxide anions to form peroxynitrite anions ($ONOO^-$), which enhance COX-2 catalytic activity in a direct manner (225). It should be noted here that NO may affect carcinogenesis in two parallel ways: first, via activation of COX-2 catalytic activity and secondly in synergy to COX-2 by inducing VEGF production (226). NO and reactive oxygen species (ROs) induce COX-2 mRNA expression (227) via the beta catenin/TCF pathway leading to activation of the polyoma enhancer activator 3 (PEA3) transcription factor (228). Moreover, NO utilizes cAMP/PKA/CREB and JNK/Jun/ATF2 signalling cascades affecting COX-2 transcription (228).

Several pro-inflammatory cytokines such as IL-1 or IFN-γ induce COX-2 expression. The major signalling pathway involved is a cAMP- and PKA-dependent activation of CREB. It is of interest that among the MAPK pathways, p38MAPK appears to be involved while ERK1/2 appears not to participate (229). On the contrary, TNF-α-induced COX-2 expression depends heavily on activation of ERK1/2 and NFkB. COX-2 expression is induced by several growth factors including IGF, TGFα and EGF. Growth factor-induced COX-2 expression takes place in both normal as well as cancer cells. The IGF-induced signals are mediated by PI3Kinase and Src/ERK but not by p38MAPK while the effect of TGF and EGF is mediated by p38MAPK, ERK1/2 and PI3K (230). UVB induces COX-2 mRNA expression in cancer cells. UVB activates the transcription factors CREB and ATF1 via a cAMP-dependent pathway. Induction of p38MAPK and PI3K but not NFkB appears to be essential for UVB-induced COX-2 expression (231). Fine-tuning of COX-2 expression levels can occur by extracellular signals such as G-protein coupled receptor (GPCR) binding proteins. The stress neuropeptides corticotropin releasing factor (CRF) and urocortins augment LPS-induced COX-2 expression via cAMP/PKA/CREB activation (232). Accumulation in the cytoplasm of this sphingosine-based lipid-signalling molecule appears to regulate COX-2 expression resulting in inflammation and tumorogenesis. It has been also implicated in the aging process. More specifically, it has been shown that ceramide is the mediator of TNF-α-induced COX-2 expression in fibroblasts and of cannabinoid-induced COX-2 expression in neuroglioma cells (233). It should be noted that

the induction of COX-2 expression by ceramide involves NFkB but not AP1 or CREB (234). Glycogen synthase kinase 3 (GSK3) phosphorylates/inactivates β-catenin leading to inhibition of the transcription factor TCF4 which regulates COX-2 transcription. Moreover, GSK3 acts through PKCδ to inactivate ERK1/2 and suppress COX-2 expression (235). Another negative regulator of COX-2 expression is the glucocorticoids. Indeed, they may represent the most important negative regulator of COX-2 transcription. They achieve this effect via inhibition of NFkB. Protein levels of COX-2 are also regulated at a prost-transcriptional level via modulation of the stability of its mRNA. COX-2 mRNA contains an ARE element at the 3′ end responsible for its stability. HuR binds on COX-2 ARE increasing its half life (236). Signals from cytokines such as IL-1β, TNF-α or TLR ligands affect COX-2 mRNA stability. The major signalling molecule mediating this process appears to be the p38MAPK. Hypoxia represents another regulator of COX-2 mRNA; it increases its stability most likely via induction of TNF-α. As mentioned above, COX-2 activity is directly regulated by NO. A recent report introduced an additional step in the regulation of COX-2 action; iNOs directly binds to COX-2 and s-nitrosylates it, thus enhancing its enzymatic activity (237) (**Fig. 12**).

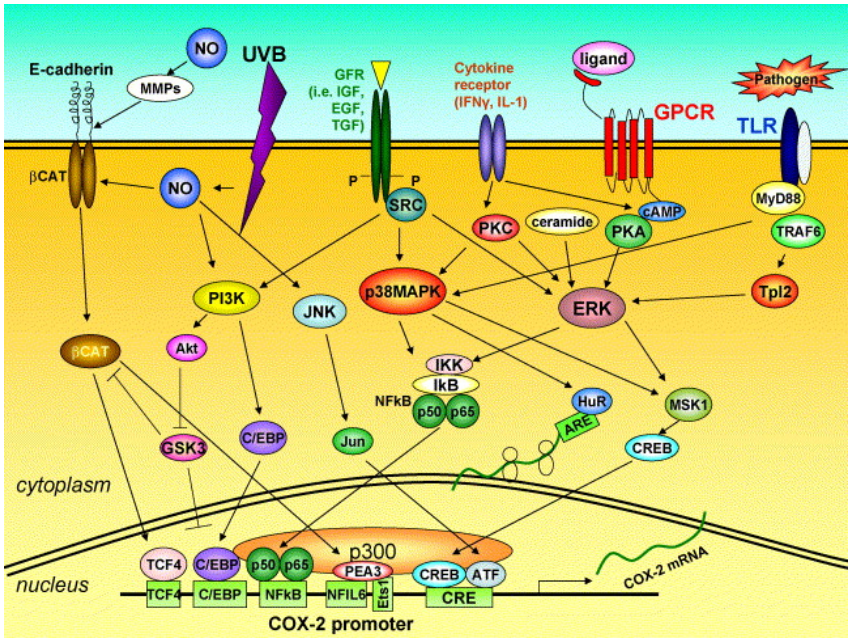


Figure 12. Signalling pathways involved in the regulation of COX-2 expression.

5.2 The cyclooxygenase-2 in diseases and cancer

COX-2 is known to be over-expressed in the synovia of patients with rheumatoid arthritis, osteoarthritis and in the colonic epithelium in ulcerative colitis. Atherosclerosis is now considered a chronic inflammatory disease, a consequence of a pathological interaction between vascular endothelial and immune cells. COX-2 is upregulated in stimulated macrophages during exposure to high levels of oxLDL and in oxLDL-induced foam cell formation (238). Selective inhibition of COX-2 expression reduces early atherogenesis in LDLR (−/−) and APO-E (−/−) mice (239). These studies suggest that COX-2 in macrophages promotes early atherosclerotic lesions through the monocyte chemotactic protein-1 (MCP1) which affects recruitment of monocytes. MCP1 is a member of the small inducible gene (SIG) family and plays a crucial role in the recruitment of monocytes to sites of injury and infection. Interestingly, lack of TLR4 reduces atherosclerosis in APO-E (−/−) mice and is associated to reduced COX-2 and MCP-1 expression in these lesions (240). COX-2 and PGE2 have been shown to promote the release and activation of MMP-2 and MMP-9 (241) which are implicated in macrophage migration and contribute to plaque rupture. Epidemiological studies suggest that anti-inflammatory drugs, especially NSAIDs, may reduce the risk for the development of Alzheimer's disease or at least delay its onset (242). It should be reminded here that the neuropathology of Alzheimer's disease is mainly characterized by deposits of amyloid beta peptides and neurofibrillary tangles as well as by a concomitant chronic inflammation which may be associated, at least in part, to the accumulation of these deposits. Thus, the beneficial effect of NSAIDs may be explained by their anti-inflammatory effects, since COX-2 expressing inflammatory cells are located around the amyloid-plaques.

Recent studies have also highlighted the relevance of COX-2 in human carcinogenesis. In fact, selective COX-2 inhibitors prevent tumorigenesis in experimental animals, and that these compounds induce apoptosis and inhibit growth in several types of cancer cells (243-246). Increased levels of COX-2 has been reported in carcinomas of the colon (247), stomach (248), esophagus (249), lung (250), liver (251), and pancreas (252). COX-2 is not

detected in normal human testes, but it is present in testicular biopsies of men with impaired spermatogenesis (253) or in testicular cancer (254).

There is increasing evidence that dysregulated expression of the COX-2 enzyme is an important pathophysiological step in breast cancer evolution, with numerous studies demonstrating increased levels of COX-2 mRNA and protein in mammary malignant tissue (255). In fact, expression of COX-2 is associated with poor prognostic features, and adverse clinical outcome (256). In human breast cancer, correlations are known to exist between COX-2 levels and expression of angiogenic factors such as vascular endothelial growth factor (257) and then development of distant metastases (258).

Moreover, it has been shown a correlation between COX-2 and aromatase expression (259) Furthermore, COX-2 inhibitors decrease aromatase mRNA and activity in breast cancer cells (260) and the use of combined COX-2 and aromatase inhibitor appear to be more effective than the single agents used alone in decreasing aromatase activity and consequently estradiol production (261).

COX-2 is considered to mediate its deleterious effects via PG synthesis. These molecules have extremely short half-lives, so direct correlation between COX-2 activity and PG presence is difficult to demonstrate experimentally. It is clear, however, that PGs stimulate cell proliferation (262), induce mitogenesis of mammary epithelial cells (263), suppress proliferation of immune cells (264). PG production is also higher in lesions associated with the presence of cancer cells in tumor lymphatics, blood vessels, and axillary nodes, and levels are greater in sites of nodal metastases compared to primary tumor areas (265).

In particular, PGE2 has been observed in high levels in breast tumor cells and is synthesized by several human breast cancer cell lines (266).

PGE2 has been found to be the most potent factor stimulating aromatase expression via promoter II (267). A correlation between COX-2 and CYP19 mRNA levels has been demonstrated in human breast cancer specimens using semi-quantitative RT-PCR (259). Furthermore, it was demonstrated that PGE2 is produced by breast tumors stimulating local estrogen biosynthesis in the surrounding adipose tissue (268;269). Local estrogen production by breast tumors is considered to be an important mechanism by which tumors

develop, evolve, and possible metastasize (268). PGs, produced through COX, exert their actions binding to specific transmembrane G-protein coupled receptors termed Prostanoid (P) receptors, present as different splice variants (270) Specifically, PGE_2 receptors, EP_1 (271), EP_2 (272), EP_3 (273) and EP_4 (274), are transduced through modulation of the activity of either adenylyl cyclase or inositol phospholipid hydrolysis and calcium mobilization (270;275). In particular, EP_1 activates PKC, EP_2 and EP_4 are coupled to PKA, EP_3 can either couple negatively to adenylyl cyclase through binding a G_i protein or associate to inositol phospholipid hydrolysis and calcium mobilization. Importantly, PGE_2 was shown to increase aromatase expression, while on the contrary EP_1 and EP_2 selective antagonists decreased the enzyme activity (276), suggesting that both PKA and PKC pathways are required for activation of promoter PII and aromatase optimal expression.

Specific aim

Several studies on both humans and rodents indicate that prenatal or postnatal exposure to estrogens migth have a central role in the mechanism leading to male reproductive tract malformations as well as testicular tumors (1;2). While the effects of estrogen on mammary gland tumorogenesis is well known, the role of aromatase overexpression and *in situ* estrogen production in testicular tumorogenesis is not clearly defined.

In this study we have investigated the molecular mechanisms causing aromatase overexpression and the effect of estradiol (E2) overproduction on Leydig cell tumor proliferation. Our hypotesis is that constitutive E2 production stimulates Leydig tumor cell proliferation acting on cell cycle regulators. Moreover, among several potential factors inducing aromatase, we investigated the role of IGF-I, produced locally in the testis, and of COX-2 overexpressed in other estrogen-dependent tumors.

Whe believe that an enhanced IGF-I production is responsible for the activation of signalling pathways determining an excess in SF-1 synthesis. This transcription factor, toghether with CREB, is the most important regulator of aromatase expression. On the other hand, we hypothesize an involvement of COX-2 in the molecular mechanism determining the constitutive activation of CREB in Leydigioma.

Materials and Methods

Cell cultures and animals

TM3 cells (immature mouse Leydig cell line) were cultured in DMEM/F-12 medium supplemented with 5% horse serum (HS), 2.5% fetal bovine serum (FBS) and antibiotics (Invitrogen, S.R.L., San Giuliano Milanese, Italy); R2C cells (rat Leydig tumor cell line) were cultured in Ham/F-10 medium supplemented with 15% HS, 2.5% FBS and antibiotics (Invitrogen, S.R.L., San Giuliano Milanese, Italy). Male Fischer 344 rats (a generous gift of Sigma-Tau Pomezia, Italy), 6 (FRN) and 24 (FRT) months of age, were used for studies. Twenty-four-month-old animals presented spontaneously developed Leydig cell tumors, which were absent in younger animals. Testes of all animals were surgically removed by qualified, specialized animal care staff in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health) and used for experiments.

Aromatase activity assay

The aromatase activity in subconfluent R2C cell culture medium was measured by tritiated water-release assay using 0.5 μM [1β-^{3}H(N)]androst-4-ene-3,17-dione (DuPont NEN, Boston, MA, USA) as a substrate (277). Incubations were performed at 37 °C for 2 h under a 95%:5% air/CO2 atmosphere. Obtained results were expressed as picomoles (pmol/h) and normalized to milligrams of protein (pmol/h/mg protein). The protein content was determined by Bradford method (278).

Radioimmunoassay (RIA)

Before the experiments, TM3 cells were maintained overnight in DMEM/F-12 and R2C cells in Ham/F-10 (medium only). The estradiol content of medium recovered from each well was determined against standards prepared in low-serum medium using a RIA kit (DSL 43100; Diagnostic System Laboratories, Webster, TX, USA). Results of the assay were normalized to the cellular protein content per well and expressed as picomoles per

milligram of cell protein. To measure IGF-I concentration in testicular extracts, testes were weighed, homogenated in 500 μL of 0.05 mol/L Tris/HCl (pH 7.6) plus protease inhibitors, and then submitted to ultrasonication followed by centrifugation, as previously published (279). IGF-I content in testicular extracts and in medium recovered from each well of R2C and TM3 cells was determined following extraction and assay protocols provided with the mouse/rat IGF-I RIA kit (DSL 2900; Diagnostic System Laboratories, Webster, TX, USA).

Chromatin immunoprecipitation (ChIP)

This assay was done using the chromatin immunoprecipitation assay kit from Upstate (Upstate Technology, Lake Placid, NY) with minor modifications in the protocol. R2C cells were grown in 100-mm plates. Confluent cultures (90%) were treated for 24 h with AG1024 (Sigma, St Louis, MO, USA), PD98059 (Calbiochem, VWR International S.R.L. Milano), LY294002 (Calbiochem, VWR International S.R.L. Milano), GF109203X (Calbiochem, VWR International S.R.L. Milano), NS398 (Sigma St Louis, MO, USA), H89 (Sigma St Louis, MO, USA) or for increasing times with 100 ng/mL IGF-I (Sigma St Louis, MO, USA), or left untreated. Following treatment, DNA/protein complexes were cross-linked with 1% formaldehyde at 37°C for 10 min. Next, cells were collected and resuspended in 400 μL of SDS lysis buffer (Upstate Technology, Lake Placid, NY) and left on ice for 10 min. Then, cells were sonicated four times for 10 s at 30% of maximal power and collected by centrifugation at 4°C for 10 min at 14,000 rpm. Ten microliters of the supernatants were kept as input (starting material, to normalize results) whereas 100 μL were diluted 1:10 in 900 μL of chromatin immunoprecipitation dilution buffer (Upstate Technology, Lake Placid, NY) and immunocleared with 80 μL of sonicated salmon sperm DNA/protein A agarose (Upstate Technology, Lake Placid, NY) for 6 h at 4°C. Immunocomplex was formed using 1 μL of 1:5 dilution of specific anti–SF-1 antibody (provided by Prof. Ken-ichirou Morohashi, Division for Sex Differentiation, National Institute for Basic Biology, National Institutes of Natural Sciences, Myodaiji-cho, Okazaki,

Japan) or using 2 µl of specific antibody anti-CREB or anti-pCREB (Cell Signaling, Celbio, Milano, Italy) overnight at 4°C. Immunoprecipitation with salmon sperm DNA/protein A agarose was continued at 4°C until the following day. DNA/protein complexes were reverse cross-linked overnight at 65°C. Extracted DNA was resuspended in 20 µL of Tris-EDTA buffer. In order to examine the binding of SF-1 and CREB, 3 µl volume of each sample and input were used for PCR using CYP19 promoter II specific primers: forward, 5'-TCAAGGGTAGGAATTGGGAC-3'; reverse, 5'-GGTGCTGGAATGGACAGATG-3'. PCR reactions were performed in the iCycler iQ Detection System (BioRad Hercules, CA, USA), using 0.1 µM of each primer, in a total volume of 50 µL reaction mixture following the manufacturer's recommendations. SYBR Green Universal PCR Master Mix (BioRad Hercules, CA, USA) with the dissociation protocol was used for gene amplification, negative controls contained water instead of DNA. Final results were calculated using the ΔΔCt method as explained above, using input Ct values instead of the 18S, calibrator was basal (untreated) sample. Amplification products were analyzed on a 1% agarose gel and visualized by ethidium bromide staining. In control samples, nonimmune rabbit immunoglobulin G was used instead of specific antibodies.

Real-time reverse transcription-PCR

Before the experiments, cells were maintained overnight in low-serum medium. Cells were then treated for the indicated times and RNA was extracted from cells using the TRizol RNA isolation system (Invitrogen, S.R.L., San Giuliano Milanese, Italy). TRizol was also used to homogenize total tissue of normal (FRNT) and tumor (FRTT) Fisher rat testes for RNA extraction. Each RNA sample was treated with DNase I (Ambion, Austin, TX), and purity and integrity of the RNA were confirmed spectroscopically and by gel electrophoresis before use. One microgram of total RNA was reverse transcribed in a final volume of 30 µL using the ImProm-II Reverse transcription system kit (Promega, Promega Italia S.R.L. Milano, Italy); cDNA was diluted 1:3 in nuclease-free water, aliquoted, and

stored at 20°C. Primers for the amplification were based on published sequences for the rat CYP19, rat CREB, and rat SF-1 genes. The nucleotide sequences of the primers for CYP19 were forward, 5'-GAGAAACTGGAAGACTGTATGGAT-3', and reverse, 5'-ACTGATTCACGTTCTCCTTTGTCA-3'. For CREB amplification, we used the following primers: forward, 5'-AATATGCACAGACCACTGATGGA-3', and reverse, 5'-TGCTGTGCGAATCTGGTATGTT-3'; for SF-1 amplification, primers have been previously published (42). PCR reactions were done in the iCycler iQ Detection System (BioRad Hercules, CA, USA) using 0.1 μmol/L of each primer, in a total volume of 30 μL reaction mixture following the manufacturer's recommendations. SYBR Green Universal PCR Master Mix (BioRad Hercules, CA, USA) with the dissociation protocol was used for gene amplification; negative controls contained water instead of firststrand cDNA. Each sample was normalized on the basis of its 18S rRNA content. The 18S quantification was done using a TaqMan rRNA Reagent kit (Applied Biosystems, Monza, Milano, Italy) following the method provided in the TaqMan rRNA Control Reagent kit (Applied Biosystems, Monza, Milano, Italy). The relative gene expression levels were normalized to a calibrator that was chosen to be the basal, untreated sample. Final results were expressed as n-fold differences in gene expression relative to 18S rRNA and calibrator, calculated using the ΔΔCt method, as follows:

$$n\text{-fold} = 2^{-(\Delta Ct_{sample} - \Delta Ct_{calibrator})}$$

where ΔCt values of the sample and calibrator were determined by subtracting the average Ct value of the 18S rRNA reference gene from the average Ct value of the different genes analyzed.

Western blot analysis

R2C and TM3 cells or total tissue of FRNT and FRTT were lysed in ice-cold radioimmunoprecipitation assay (Ripa) buffer containing protease inhibitors (20 mmol/L Tris, 150 mmol/L NaCl, 1% Igepal, 0.5% sodium deoxycholate, 1 mmol/L EDTA, 0·1% sodium dodecyl sulphate (SDS), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 0.15 units/ml aprotinin and 10 μmol/L leupeptin) for protein extraction. The protein content was determined by Bradford method (278). The proteins were separated on 11% SDS/polyacrylamide gel and then electroblotted onto a nitrocellulose membrane. Blots were incubated overnight at 4 °C with: 1. anti-human P450 aromatase antibody (1:50) (Serotec, Oxford, UK, MCA 2077), 2. anti-ERα (F-10) antibody (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc8002), 3. anti-ERβ (H-150) (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc8974), 4. anti-cyclin D1 (M-20) antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc718), 5. anti-cyclin E (M-20) antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc481), 6. anti-CREB antibodies (1:1000) (Cell Signaling Technology, Celbio, Milan, Italy, 48H2) and (1:1000) (Biosource Inc. Camarillo CA USA, AHO0842); 7. anti-pCREB ser133 (1:1000) (Cell Signaling Technology, Celbio, Milan, Italy, 87G3) or anti-pCREB Ser129/133 (1:1000) (Biosource Inc. Camarillo CA USA, 44-297G), 8. anti SF-1 (1:1000) provided by Prof. Ken-ichirou Morohashi, Division for Sex Differentiation, National Institute for Basic Biology, National Institutes of Natural Sciences, Myodaiji-cho, Okazaki, Japan), 9. anti-pSF-1 (1:1000) provided by Dr Holly A. Ingraham Department of Physiology, University of California, San Francisco, San Francisco, California 94143-0444, USA), 10. anti–IGF-I receptor (IGF-IR; 1:800; Santa Cruz Biotechnology Santa Cruz, CA, USA, sc713; 11. anti COX-2 (1:1000) (Cell Signaling Technology, Celbio, Milan, Italy, 4842), 12. anti-actin (C-2) antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc8432), 13. anti GAPDH (FL-335) (1:3000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc25778). Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) and

immunoreactive bands were visualized with the ECL western blotting detection system (Amersham Biosciences, Cologno Monzese, Italy). To assure equal loading of proteins membranes were stripped and incubated overnight with β-actin or GAPDH antiserum.

Immunohistochemical analysis

Tumor human testicular tissues were retrieved from biopsy of two male patients with Leydig cell tumour (ages 31 and 33 years). Control human testicular tissues were retrieved from biopsy of two male patients (ages 29 and 35 years) showing testes with a Sarcoidosis-like granulomatous lesion. The archival human cases were provided by the Pathologic Anatomy Unit (Annunziata, Hospital) Italy.

Immunohistochemical experiments were performed on formalin–fixed and paraffin-embedded testis tissues after heat-mediated antigen retrieval (280). Paraffin embedded sections, 5μm thick, were mounted on slides precoated with poly-lysine, and then they were deparafinized and dehydrated (7-8 serial sections). Hydrogen peroxide (3% in distillate water) was used, for 30 minutes, to inhibit endogenous peroxidase activity while normal horse serum (10%) was utilised, for 30 minutes, to block the non-specific binding sites.

Immunodetection was carried out using anti-COX2 (1:50), primary antibodies at 4°C overnight. Then, a biotinylated horse universal IgG was applied (1:600) for 1 hour at RT, followed by the avidin-biotin-horseradish peroxidase complex (ABC/HRP) (Vector, Laboratories, CA, USA). Immunoreactivity was visualized by using the diaminobenzidine chromogen (DAB)(Zymed Laboratories, CA, USA). Sections were also counterstained with haematoxylin. The primary antibody was replaced by normal rabbit serum in negative control sections. Absorption controls have utilised primary antibodies preabsorbed with an excess (5nmol/ml) of the purified respective blocking peptides at 4°C for 48 hours (data not show).

Cell proliferation assay

For proliferative analysis a total of $1x10^5$ cells were seeded onto 12-well plates in complete medium and let grow for 2 days. Prior to experiments, cells were maintained for 24h in Ham/F-10 medium and the day after treated with ICI 182780 (Astra-Zeneca, Basiglio, Milano, Italy), 4-hydroxytamoxifen (OHT) (Sigma St Louis, MO, USA), Letrozole (Novartis Pharma AG, Basel, Switzerland), NS398 (Sigma, St Louis, MO, USA), H89 (Sigma, St Louis, MO, USA), AH 23848 (Sigma, St Louis, MO, USA), 17β-estradiol (E2) (Sigma St Louis, MO, USA), or treated at the indicated times with IGF-I or PGE2 (Sigma, St Louis, MO, USA), alone or in combination with inhibitors or incubated with an anti IGF-I antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc1422). Control (basal) cells were treated with the same amount of vehicle alone (DMSO) that never exceeded the concentration of 0.01% (v/v). [^{3}H]Thymidine incorporation was evaluated after 6 h incubation with 1 μCi [^{3}H]thymidine (PerkinElmer Life Sciences, Boston, MA, USA) per well. Cells were washed once with 10% trichloroacetic acid (TCA) (Sigma, St Louis, MO, USA), twice with 5% trichloroacetic acid and lysed in 1 ml 0.1 mol/L NaOH at 37°C for 30 min. The total suspension was added to10 ml optifluor fluid and was counted in a scintillation counter.

Non-radioactive in vitro assay for PKA activity in cell lysates

This assay was performed using the PepTag for non-radioactive detection of cAMP-dependent protein kinase assay kit from Promega (Madison, WI). R2C cells were grown in 100X60mm plates to 100% confluence. After stimulation (4h) cells were washed with phosphate-buffered saline (PBS) (5ml per 100 mm disch) and lysed in cold PKA extraction buffer (0.5 ml/plate) containing 25 mmol/L Tris–HCl pH 7.5, 0,5 mmol/L EDTA, 0,5mol/L EGTA, 10mmol/L beta–mercaptoethanol, 1ug/ml leupeptin, 1ug/ml aprotinin. The lysates were cleared by centrifugation at 14,000×g, 5 min and 5 ul of cleared lysates were subjected to a kinase reaction with the fluorescence-labeled PKA substrate, kemptide,

following the manufacturer's protocol. The reaction was stopped by boiling the samples for 10 min. The samples were separated on 0,8% agarose gel by electrophoresis at 100V for 15 minutes. Phosphorylated peptide migrated toward the anode (+), while non-phosphorylated peptide migrated toward the cathode (-). The gel was photographed on a transilluminator. The quantitative differences in the amount of phosphorylated and non-phosphorylated peptide species were detected by spectrophotometric method reading the absorbance at 570 nM.

RNA interference

COX-2 Sthealth® siRNA and scrambled siRNA were purchased from Invitrogen (Invitrogen, S.R.L., San Giuliano Milanese, Italy). Twenty-four hours after plating cells into 60 mm dishes at 4×10^6 cells, siRNAs were transfected to a final concentration of 50, 100 or 200 nM using Lipofectamine2000 Transfection Reagent (Invitrogen, S.R.L., San Giuliano Milanese, Italy) according to manufacturer's instructions. Two days after transfection cells were used for western analysis or cell proliferation assay.

.

Data Analysis and Statistical Methods

Pooled results from triplicate experiments were analyzed using one-way ANOVA with Student-Newman-Keuls multiple comparison methods, using SigmaStat version 3.0 (SPSS, Chicago, IL).

Results

Estradiol induces Leydig cell tumor proliferation through an autocrine mechanism

We performed our study utilizing as model system R2C Leydig tumor cells. These cells have been demonstrated to have high aromatase expression and, consequently, activity (40), while we used another Leydig cell line, TM3 cells, as a normal control. We also analyzed testes from older and younger Fischer rats. Aged animals have a high incidence of spontaneous neoplasm of Leydig cells (281;282), a phenomenon not observed in younger animals, allowing us to use them as a good *in vivo* model to confirm results obtained in cell lines. Our first step was to measure estradiol (E2) content in culture medium of R2C and TM3 cells maintained in culture for increasing time. While E2 levels in TM3 medium were extremely low (data not shown) in R2C cells E2 levels after 24 h were 0.5 pmol/mg protein and increased by 7-fold at 96 h (Fig. 1A).

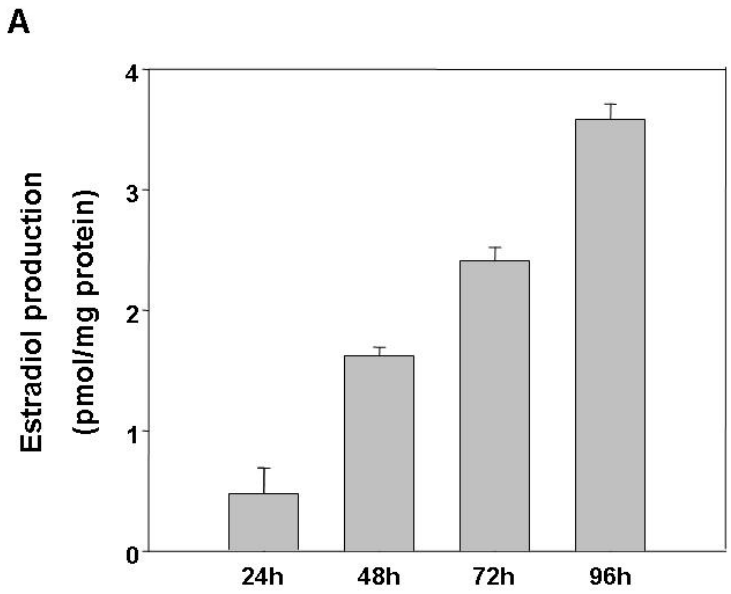


Figure 1 A. E2 production in R2C cells. R2C cells were cultured for the indicated times in serum free medium. E2 content was determined by RIA and normalized to the cell culture well protein content. Data represent the mean ± SEM of values from three separate cell culture wells expressed as pmol/mg protein.

This production was dependent on high constitutive active aromatase activity, since the presence of aromatase inhibitor Letrozole was able to decrease E2 production at all time points tested (Fig. 1B). E2 levels after 24 h treatment with Letrozole were still detectable, but were completely knock down when we removed the medium after 24 h and renewing the treatment for an additional 24 h. The same effect was maintained for the other two time points investigated (Fig. 1B).

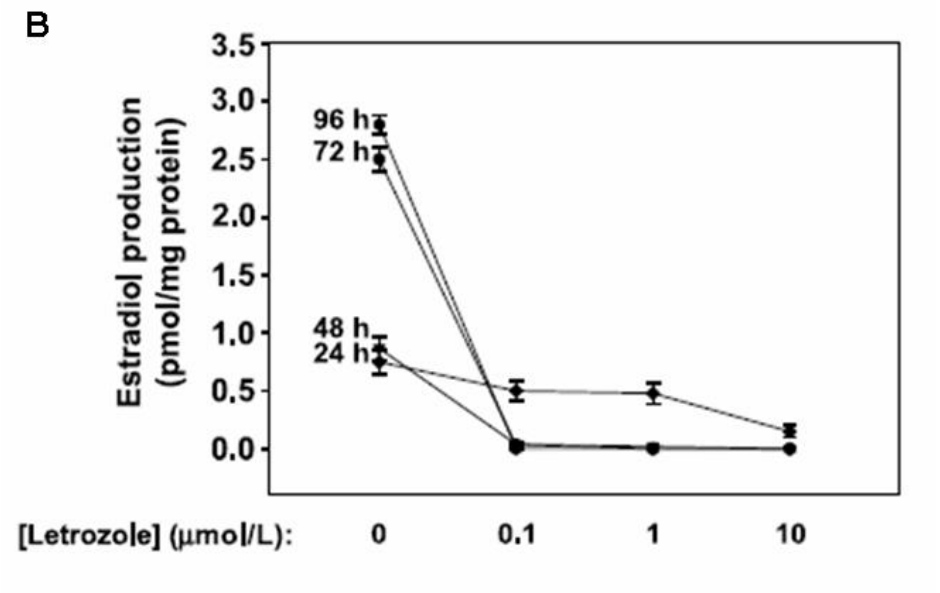


Figure 1 B. E2 production in R2C cells. R2C cells were treated for the indicated times in HAM-F10 in the absence (0) or presence of aromatase inhibitor letrozole (0.1, 1, 10 µmol/L). Every 24h, before renewing treatment, cell culture medium was removed and analyzed for steroid content. E2 content was determined by RIA and normalized to the cell culture well protein content. Data represent the mean ± SEM of values from three separate cell culture wells expressed as pmol/mg protein.

Once estradiol is produced it can exert its actions binding to specific receptors, the estrogen receptors α e β (ERα and ERβ). Analysis of the two receptor protein isoforms in our models demonstrated that tumor Leydig cells express both isoform of ERs (Fig. 2). Particularly the α isoform seems to be more expressed in R2C cells respect to TM3 and in FRTT respect to the its control FRNT where ERβ is more expressed. Moreover aromatase protein content is extremely high in tumoral samples(139) (Fig. 2).

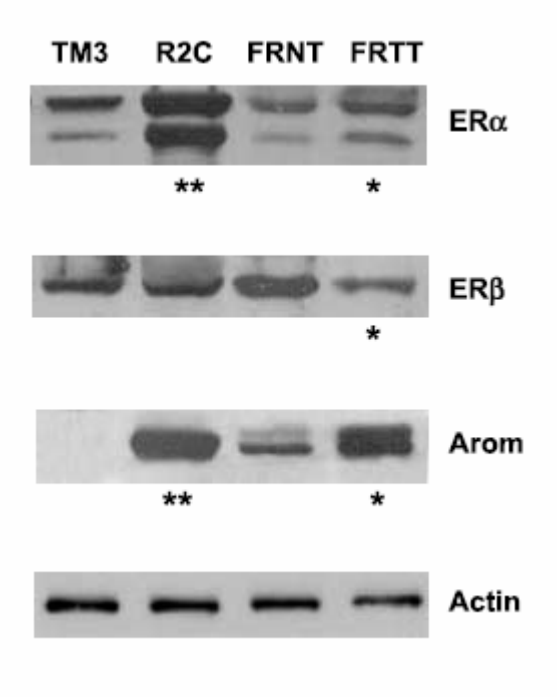


Figure 2. Expression of estrogen receptors (ER) and aromatase in R2C cells. ERα , ERβ and aromatase western blot analysis was performed on 50 μg of total proteins extracted from TM3 and R2C cells or from total tissue of normal (FRNT) and tumor (FRTT) Fisher rat testes. Results are representative of three independent experiments. β-Actin was used as a loading control. Protein expression in each lane was normalized to the β-actin content and expressed as fold over control represented by normal cells. Normalized absorbances were subjected to statistical analysis; statistically significant differences are indicated (*, $P < 0.05$; **, $P < 0.01$, compared with control).

Our next experiments demonstrated that estrogen receptors are required for proliferation through a short autocrine loop maintained by endogenous E2 production in Leydig tumor cells.

For instance, the use of both antiestrogens hydroxytamoxifen (OHT) and ICI 182,760 (ICI) and the use of aromatase inhibitor Letrozole (Letr) determined a dose-dependent inhibition of cell proliferation (Fig. 3A). Among the different doses tested the highest dose of OHT (10 μmol/L) was able to inhibit cell proliferation by 90%, ICI (10 μmol/L) by 86% and letrozole (10 μmol/L) by 70%.

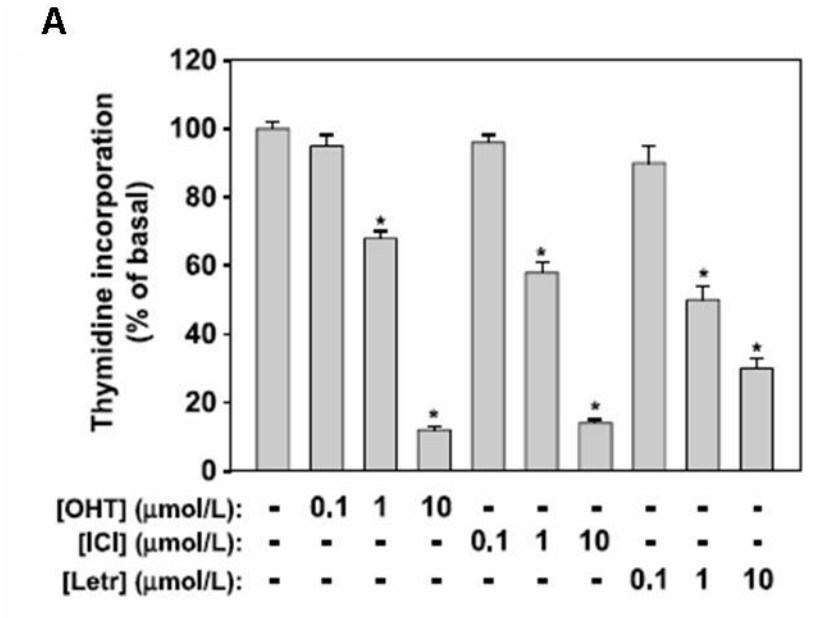


Figure 3 A. Effects of antiestrogens, aromatase inhibitor Letrozole and estradiol on R2C cell proliferation. Cells were treated for 96h in HAM-F10 in the absence (-) or presence of antiestrogens hydroxytamoxifen (OHT) or ICI 182,760 (ICI) or aromatase inhibitor letrozole (Letr) at the indicated concentrations. Proliferation was evaluated by [3H] Thymidine incorporation analysis. Values expressed as percent of untreated (basal) cells (100%) represent the mean ± SEM of three independent experiments each performed in triplicate. (*P< 0.05 compared with basal condition).

In the same vein, starving cells for prolonged time and changing the medium every day in order to remove local E2 production, we found that addition of 1, 10 and 100 nmol/L E2 stimulated Leydig tumor cell proliferation (Fig. 3B), and partially abrogated the inhibition induced by Letrozole (Fig. 3C).

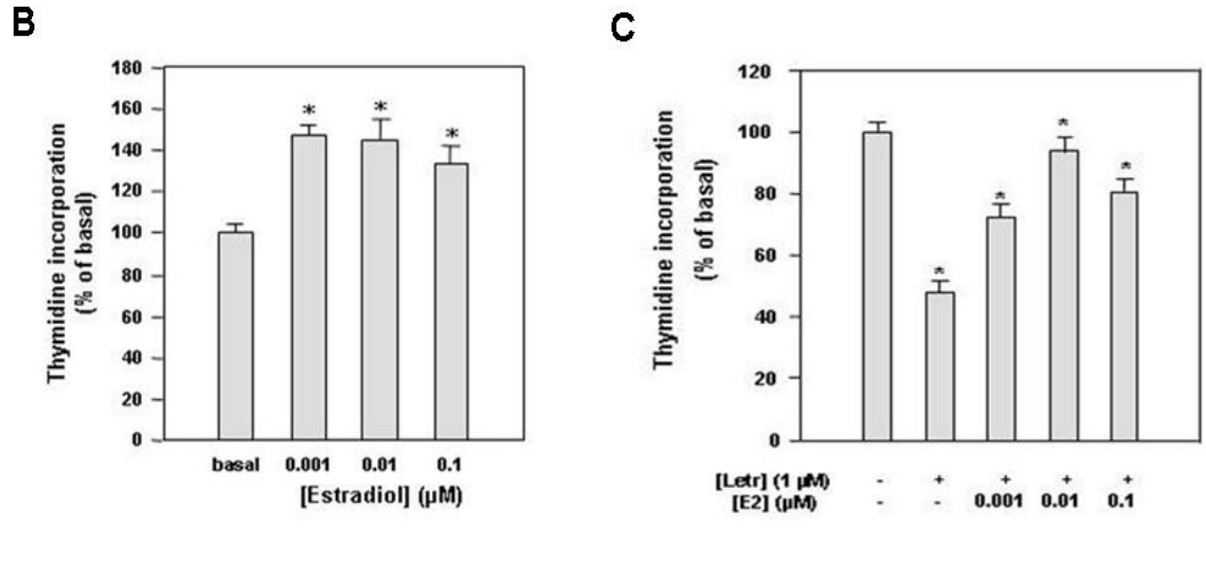


Figure 3 B, C. Effects of antiestrogens, aromatase inhibitor Letrozole and estradiol on R2C cell proliferation. (**B**) Cells were cultured for 48h in serum-free HAM-F10, every 24 h cell culture medium was removed and renewed. Cells were then treated for 24 h with estradiol at the indicated concentrations. (**C**) Cells were cultured for 24h in serum-free HAM-F10, cells were then treated for 48 h with letrozole (1µmol/L) changing the culture medium and renewing treatment every 24h. For additional 24h cells were treated with letrozole (Letr) (1µmol/L) in combination with estradiol (E2) at the indicated concentrations. Proliferation was evaluated by [3H] Thymidine incorporation analysis. Values expressed as percent of untreated (basal) cells (100%) represent the mean ± SEM of three independent experiments each performed in triplicate. (*P< 0.05 compared with basal condition).

The stimulatory effect of E2 was concomitant with the increased levels of cell cycle regulator cyclin D1 and E, whose expression was inhibited by pure antiestrogen ICI 182,760 (Fig. 4). All these results address how the classic E2/ERα signalling may control Leydig cell tumor growth and proliferation similarly to what observed in other estrogen-dependent tumors.

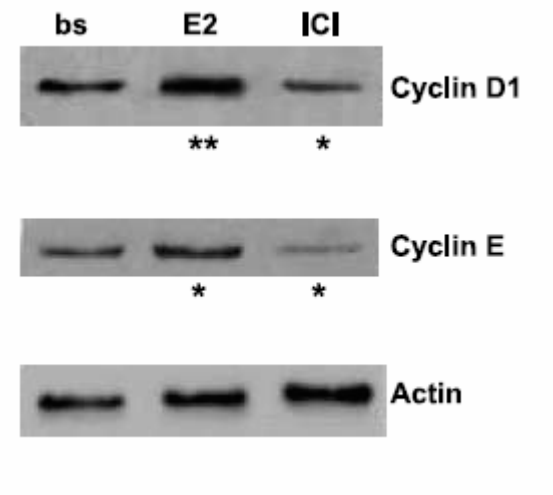


Figure 4. Effects of E2 and ICI 182,760 on cyclin D1 and E protein levels. R2C cells were cultured for 48h in serum-free HAM-F10, every 24 h cell culture medium was removed and renewed. Cells were then treated for 24 h in the absence (bs) or in the presence with estradiol (E2) (1 nmol/L) and ICI 182,760 (ICI) (1 μmol/L) before extracting total proteins. Western blot analysis of Cyclin D1 and Cyclin E was performed on 50 μg of total proteins extracted from R2C cells. Blots are representative of three independent experiments with similar results. β-Actin was used as a loading control. Protein expression in each lane was normalized to the β-actin content and expressed as fold over control represented by basal condition. Normalized absorbances were subjected to statistical analysis; statistically significant differences are indicated (*, $P < 0.05$; **, $P < 0.01$, compared with basal condition).

Aromatase overexpression is determined by constitutive activation of transcription factors SF-1 and CREB

Aromatase gene transcription in rat Leydig cells is driven by the PII promoter, which is principally regulated through three CRE-like sites and one NRE site binding SF-1 and LRH-1 (40;42). Constitutive active levels of CREB have been previously demonstrated in R2C cells (283).

Here we confirmed these data and demonstrated high phosphorylated status of CREB together with enhanced phosphorylation of SF-1 in FRTT (Fig. 5). Furthermore we demonstrated the presence of high expression levels of SF-1 with the protein present in a phosporylated status in R2C but not in TM3.

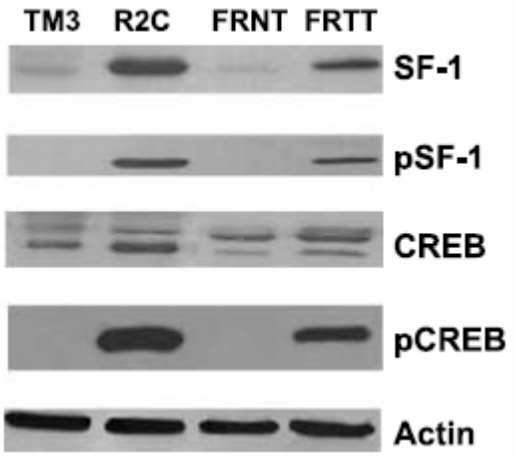


Figure 5. Expression of total and phosphorylated forms of SF-1 and CREB. Western blot analyses of SF-1, pSF-1, CREB, and pCREB were done on 50 μg of total proteins extracted from TM3 and R2C cells or from total tissue of normal (FRNT) and tumor (FRTT) Fisher rat testes. Blots are representative of three independent experiments with similar results. β-actin was used as loading control.

IGF-I is produced by R2C cells and induces aromatase expression through PI3K- and PKC- mediated activation of SF-1

Starting from previous findings showing the ability of IGF-I to activate SF-1 and CREB, which leads to an increase in StAR transcription and then steroidogenesis (3;284), we investigated the role of this factor in regulating aromatase.

Determination of IGF-I content in TM3 and R2C culture medium by RIA revealed a significant difference in growth factor production, with R2C cells producing 4-fold higher IGF-I amounts (Fig. 6A). Moreover, we measured IGF-I content in testicular tissues, revealing a significant difference between FRTT and FRNT (Fig. 6B).

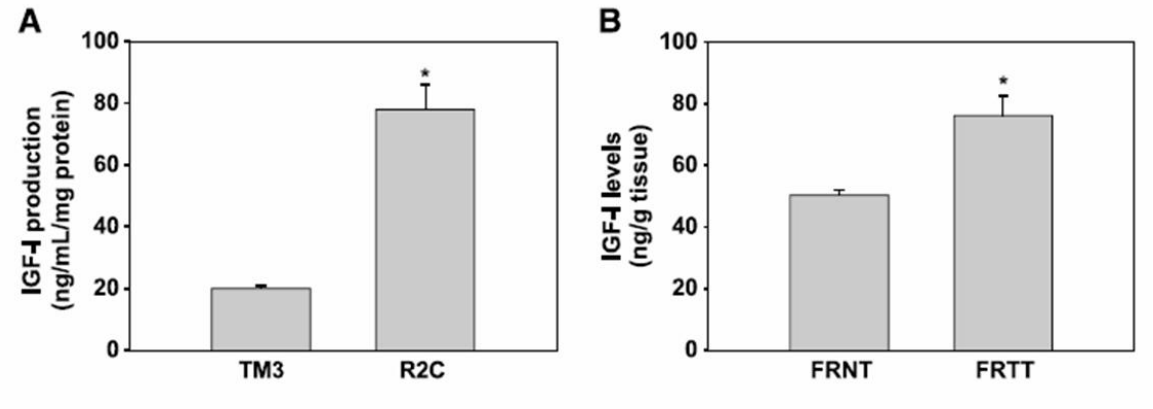


Figure 6 A, B. **IGF-I production and autocrine effects in Leydig cells.** (**A)** TM3 and R2C cells were cultured for 24 h in serum-free medium and IGF-I levels in culture medium were determined by RIA. IGF-I levels were normalized to the cell culture well protein content. *Columns*, mean of three independent experiments each done in triplicate; *bars*, SE. (**B)** Total protein extracts from FRNT and FRTT were assayed for IGF-I content. IGF-I levels were normalized to the tissue weight. *Columns*, mean of three independent samples; *bars*, SE. *, $P < 0.01$, compared with control conditions, represented by TM3 cells or FRNT.

IGF-I exerts its actions by binding to specific receptors (IGF-IR); however, we did not reveal differences in IGF-IR expression between TM3 and R2C cells (Fig. 6C). Upon binding to its receptor, IGF-IR, IGF-I activates three major transductional pathways: Ras/Raf/MAPK, PI3K/AKT, PLC/PKC; to demonstrate involvement of IGF-I transductional pathways in modulating aromatase expression in Leydig cell tumors, we used specific inhibitors: of IGF-I receptor (IGF-IR) [AG1024 (AG)], of ERK1/2 [PD98059 (PD)], of PI3K [LY294002 (LY)] and of PKC [GF109203X (GFX)]. IGF-I receptor inhibitor (AG1024) was able to inhibit aromatase activity in R2C cells by 85%, LY294002 determined 65 % inhibition, PD98059 35 % and GF109203X 61% (Fig. 6D).

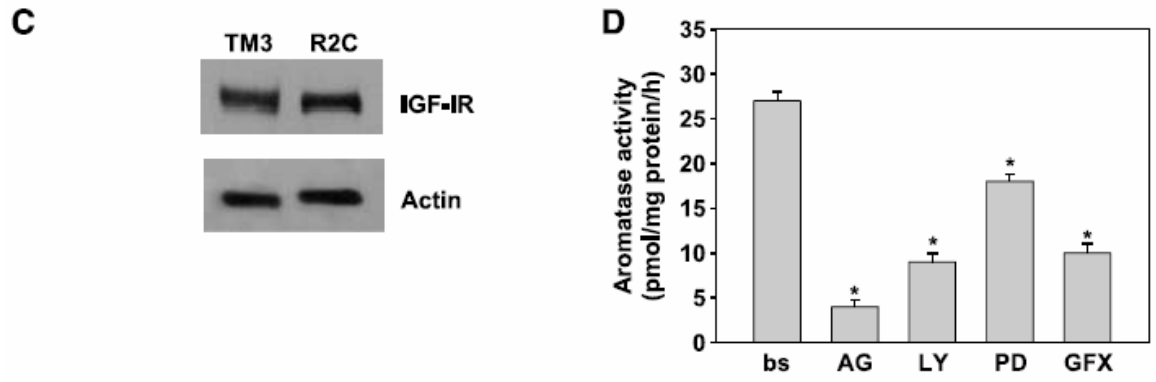


Figure 6 C, D. **IGF-I production and autocrine effects in Leydig cells.** (**C)** Western blot analysis of IGF-IR in TM3 and R2C cells. β-Actin was used as a loading control. (**D)** R2C cells were treated with AG1024 (AG; 20 μmol/L), LY294002 (LY; 10 μmol/L), PD98059 (PD; 20 μmol/L), and GF109203X (GFX; 20 μmol/L). Aromatase activity was assessed by using the modified tritiated water method. Results obtained are expressed as picomoles of [3H]H2O released per hour and normalized to the well protein content (pmol/h/mg protein). *Columns*, mean of three independent experiments each done in triplicate; *bars*, SE. *, $P < 0.01$, compared with basal (bs).

The same inhibitory pattern was observed also on aromatase mRNA (Fig. 7A). Parallely all of the different inhibitors but not PD98059 were able to reduce SF-1 mRNA (Fig. 7 B), while CREB remained unchanged (Fig. 7C). For SF-1 inhibition was 75% with AG1024, 90 % with LY204002 and 80 % with GF109203X (Fig. 7B).

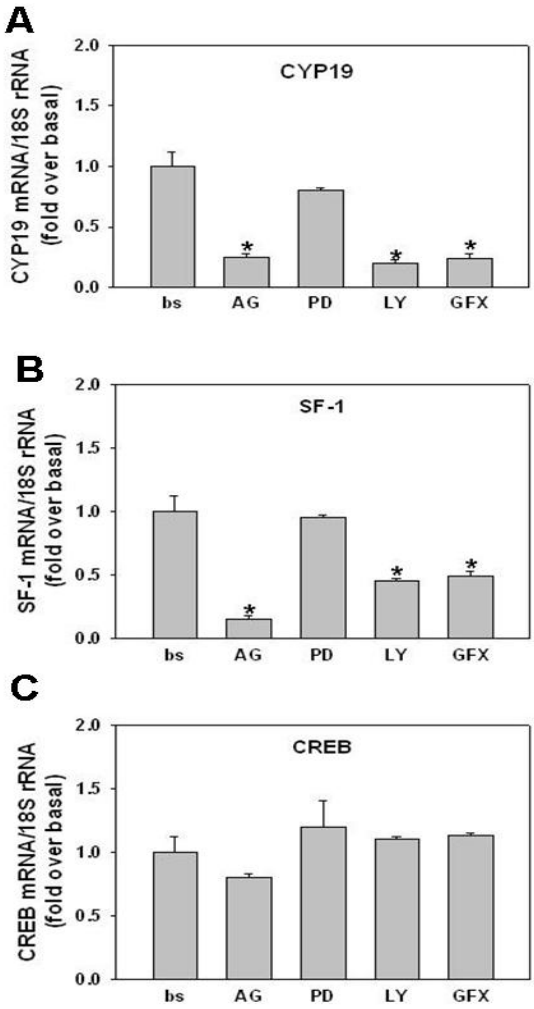


Figure 7. Effects of inhibitors of IGF-I pathways on mRNA expression of CYP19, SF-1 and CREB in R2C cells. Total RNA was extracted from R2C cells untreated (bs) or treated for 24h with AG1024 (AG) (20µmol/L), LY294002 (LY) (10 µmol/L), PD98059 (PD) (20 µmol/L) and GF109203X (GFX) (20 µmol/L). Real time RT-PCR was used to analyze mRNA levels of CYP19, SF-1, and CREB. Data represent the mean ± SEM of values from three separate RNA samples. Each sample was normalized to its 18S ribosomal RNA content. Final results are expressed as n-fold differences of gene expression relative to calibrator (bs) calculated with the ΔΔCt method. * $P < 0.001$ compared to basal condition (bs).

Analysis of protein levels by western blot confirmed the data on mRNA (Fig. 8). Treatments with increasing doses of AG1024 (Fig. 8A), LY204002 (Fig. 8B) and GF109203X (Fig. 8D) but not PD98059 (Fig. 8C) were able to induce a dose-dependent inhibition of total and phosphorylated levels of SF-1, on the other hand CREB was not affected by the presence of any of the inhibitors (Fig. 8).

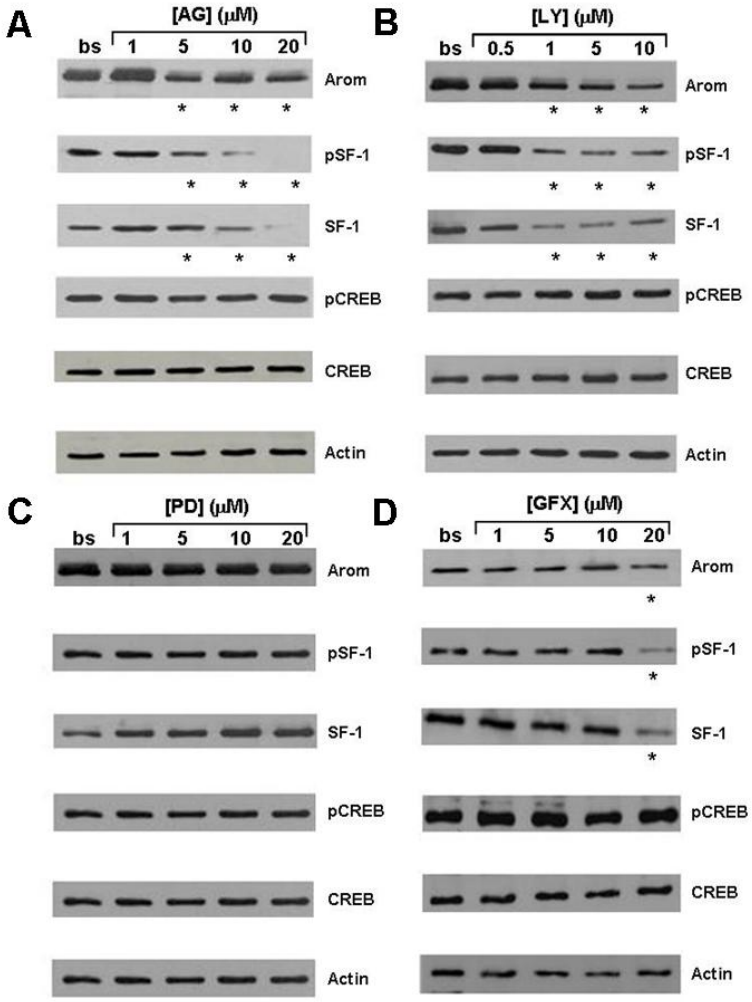


Figure 8. Effects of IGF-I pathway inhibitors on aromatase, SF-1 and CREB expression in R2C cells. A to **D**, Western blot analyses were done on 50 μg of total proteins extracted from R2C cells untreated (bs) or treated for 24 h with the indicated doses of AG1024 (AG) **(A)**, LY294002 (LY) **(B)**, PD98059 (PD) **(C)**, and GF109203X (GFX) **(D)**. Representative of three independent experiments with similar results. β-Actin was used as a loading control. Normalized absorbances were subjected to statistical analysis; statistically significant differences are indicated (*, $P < 0.01$, compared with basal).

IGF-I induces aromatase expression and activity in R2C cells

To further demonstrate the prevalent role of SF-1 in IGF-I induced aromatase expression in Leydig cell tumor, we monitored the effect of IGF-I on CYP19 and SF-1 expression. Addition of exogenous amounts of IGF-I were able to induce aromatase activity by 1.8-fold (Fig. 9).

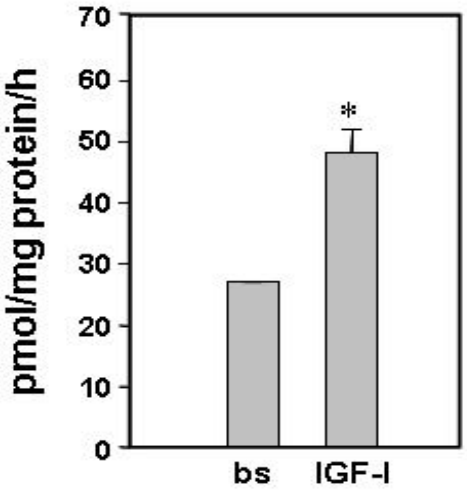


Figure 9. Aromatase activity in R2C cells in response to IGF-I. Cells were treated with IGF-I (100 ng/ml) for 24h. Aromatase activity was assessed by using the modified tritiated water method. Results obtained are expressed as pmoles of [^{3}H]H2O released per hour and are normalized to the well protein content (pmol/h/mg protein). Values represent the mean ± SEM of three independent experiments each performed with triplicate samples. *P<0.05 compared to basal (bs).

A significant effect of IGF-I treatment was seen also on CYP19 mRNA levels (Fig. 10A). IGF-I was able to induce a significant increase of 2- and 3.8-fold in aromatase mRNA at 12h and 24h, respectively (Fig. 10A). Aromatase protein levels under the same treatments reflected the mRNA data (Fig. 10B). Analysis of expression levels of total and phosphorylated forms of transcription factors SF-1 and CREB showed an increase in SF-1 and pSF-1 in the presence of IGF-I starting at 4 h, whereas no differences were observed for CREB at any of the investigated times (Fig. 10B).

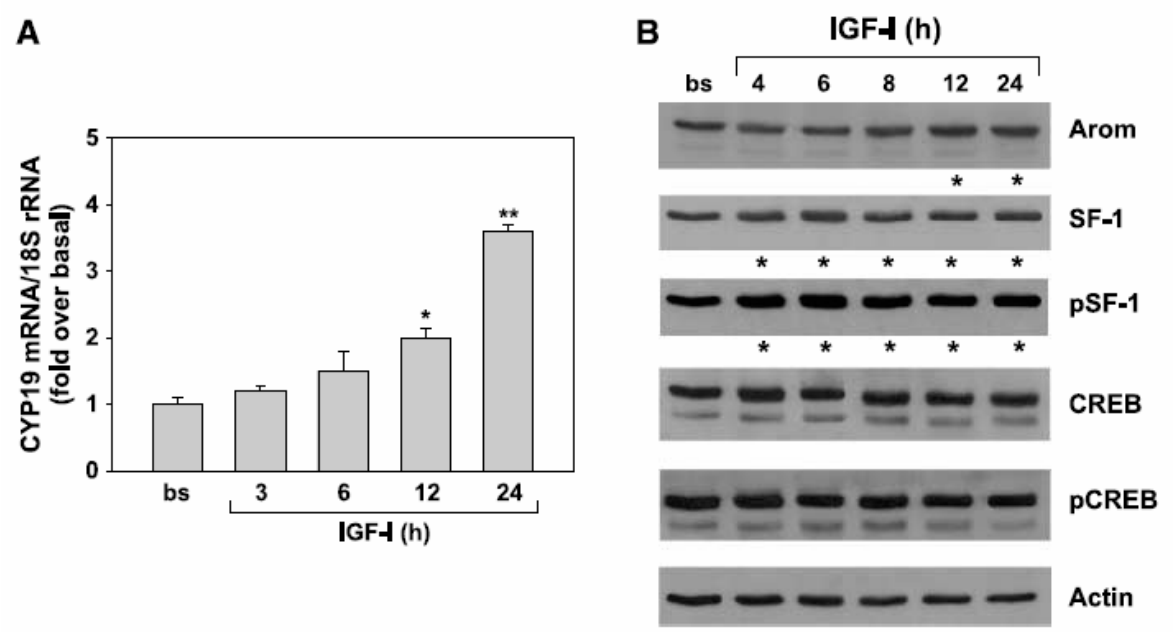


Figure 10 A, B. Effects of IGF-I on aromatase, SF-1 and CREB expression in R2C cells. Cells were treated in serum-free medium for the indicated times with IGF-I (100 ng/mL). (**A),** Total RNA was extracted from R2C cells untreated or treated as indicated. Real-time reverse transcription-PCR was used to analyze CYP19 mRNA levels. *Columns*, mean of values from three separate RNA samples; *bars*, SE. Each sample was normalized to its 18S rRNA content. *, $P < 0.01$; **, $P < 0.001$, compared with basal. (**B)**, Western blot analyses were done on 50 μg of total proteins extracted from R2C cells untreated (bs) or treated as indicated. Representative of three independent experiments with similar results. β-Actin was used as a loading control. Normalized absorbances were subjected to statistical analysis; statistically significant differences are indicated (*, $P < 0.01$, compared with bs).

AG1024, LY294002, and GF109203X were able to inhibit IGF-I effects on CYP19 mRNA (Fig. 10C) and protein levels (Fig. 10D) as a consequence of a decreased SF-1 expression (Fig. 10D).

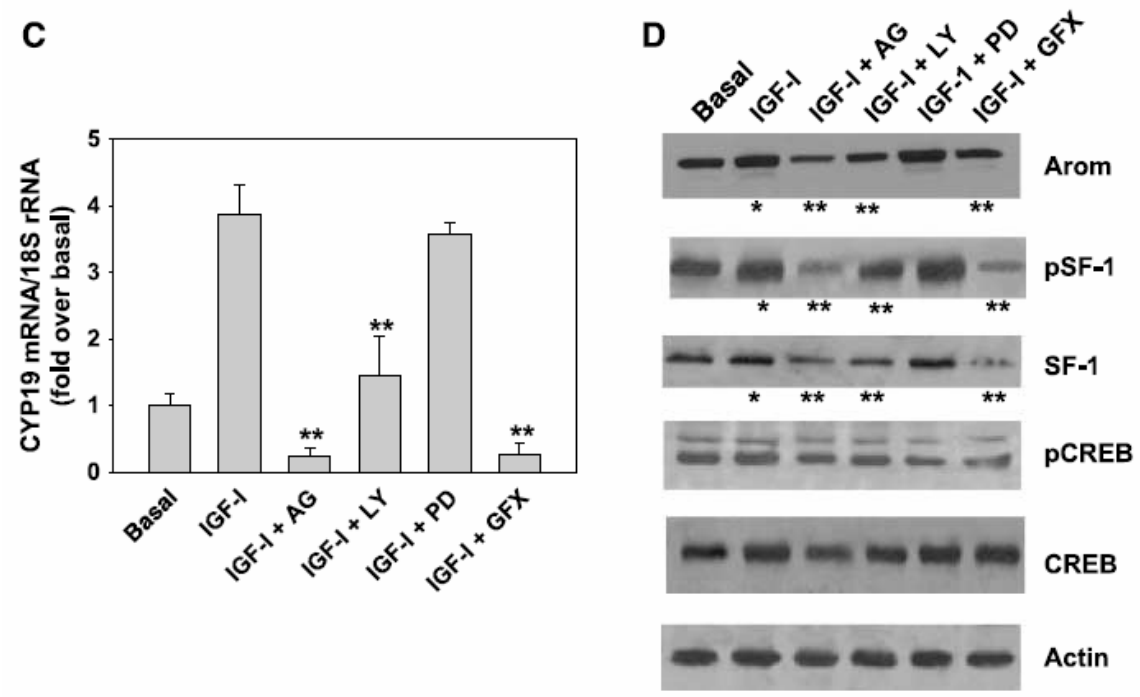


Figure 10 C, D. Effects of IGF-I on aromatase, SF-1 and CREB expression in R2C cells. Cells were treated in serum-free medium for 24 h with AG1024 (AG) (20 μmol/L), LY294002 (LY) (10 μmol/L), PD98059 (PD) (20 μmol/L), and GF109203X (GFX) (20 μmol/L), alone or in combination with IGF-I (100 ng/mL). (**C)**, Total RNA was extracted from R2C cells untreated or treated as indicated. Real-time reverse transcription-PCR was used to analyze CYP19 mRNA levels. *Columns*, mean of values from three separate RNA samples; *bars*, SE. Each sample was normalized to its 18S rRNA content. **, $P < 0.001$, compared with IGF-I. (**D)**, Western blot analyses were done on 50 μg of total proteins extracted from R2C cells untreated (basal) or treated as indicated. Representative of three independent experiments with similar results. β-Actin was used as a loading control. Normalized absorbances were subjected to statistical analysis; statistically significant differences are indicated (*, $P < 0.01$, compared with basal; **, $P < 0.01$, compared with IGF-I).

Changes in IGF-I pathway activation status lead to changes in SF-1 binding to the aromatase PII promoter

We also performed CHIP assay to investigate how IGF-I stimulation influence per se binding of transcription factors to the aromatase PII promoter. We evidenced how in basal condition all the different inhibitors but not PD98059 reduced the amount of bound SF-1 reflecting changes in SF-1 protein amount (Fig. 11A). The increase in SF-1 protein content seen under IGF-I treatment (Fig. 10B) reflected an increase in SF-1 binding to the PII promoter (Fig. 11B).

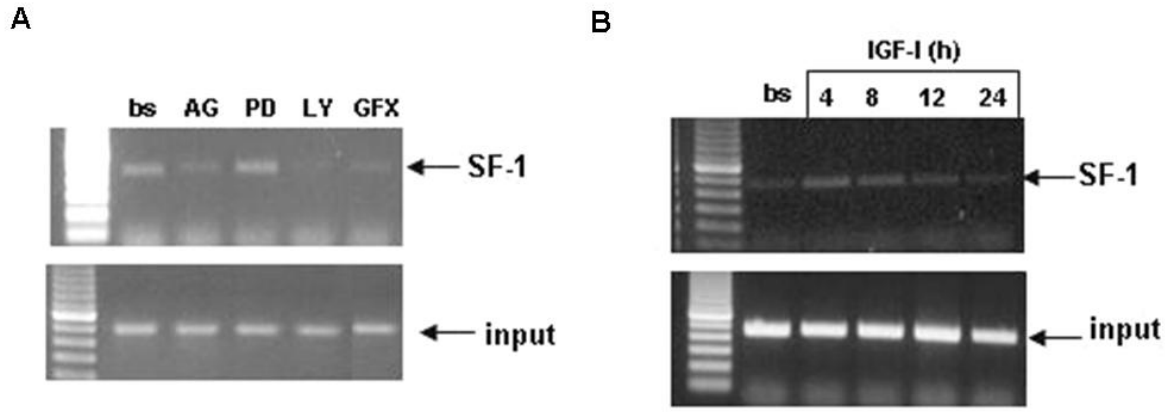


Figure 11. IGF-I increases SF-1 recruitment to the aromatase PII promoter through PI3K and PKC. (A) R2C cells were incubated for 24 h with AG1024 (20μmol/L), LY294002 (10 μmol/L), PD98059 (20 μmol/L) and G109203FX (20 μmol/L). Untreated cells (bs) were treated with the same amount of vehicle alone (DMSO) that never exceeded 0·01% (v/v). **(B)** R2C cells were incubated for the indicated times with IGF-I (100 ng/ml). *In vivo* binding of SF-1 to the aromatase PII promoter was examined using ChIP assay. Immunoprecipitated (SF-1) and total (10% input) DNA were subject to PCR using specific primers. Similar results were obtained in two additional experiments.

IGF-I–induced estradiol production modulates R2C cell proliferation

Treatment with IGF-I induces aromatase activity and estradiol production, which are decreased by AG1024, LY294002, and GF109203X, as well as by PD98059 (Fig. 12A and B).

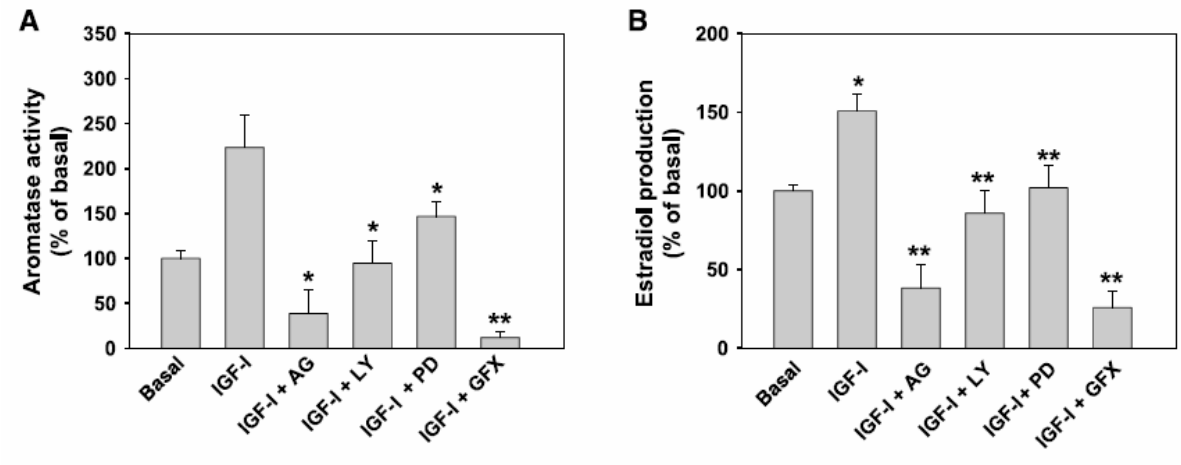


Figure 12 A, B. Effects of IGF-I and IGF-I pathway inhibitors on estradiol production and R2C cell proliferation. Cells were treated in serum-free medium for 24 h with IGF-I (100 ng/mL)alone or in combination with AG1024 (AG) (20 μmol/L), LY294002 (LY) (10 μmol/L), PD98059 (PD) (20 μmol/L), and GF109203X (GFX) (20 μmol/L). **(A)**, Aromatase activity is expressed as percent of basal. *Columns*, mean of three independent experiments each done in triplicate; *bars*, SE. *, $P < 0.01$; **, $P < 0.001$, compared with IGF-I. **(B)**, R2C cells were maintained for 48 h in serum-free medium, before being treated for 24 h. E2 content in R2C culture medium was determined by RIA and normalized to the cell culture well protein content. *Columns*, mean percent of basal; *bars*, SE. *, $P < 0.05$, compared with basal; **, $P < 0.01$, compared with IGF-I.

The observed changes in estradiol production modified R2C cell proliferative behavior (Fig. 12C). In addition, the use of an anti–IGF-I antibody in immunoneutralization experiments caused a dose-dependent inhibition in tritiated thymidine incorporation (Fig. 12C). The ability of IGF-I to stimulate, and that of the inhibitors to block, cell proliferation was linked to an alteration in cyclin D1 and cyclin E expression (Fig.12D).

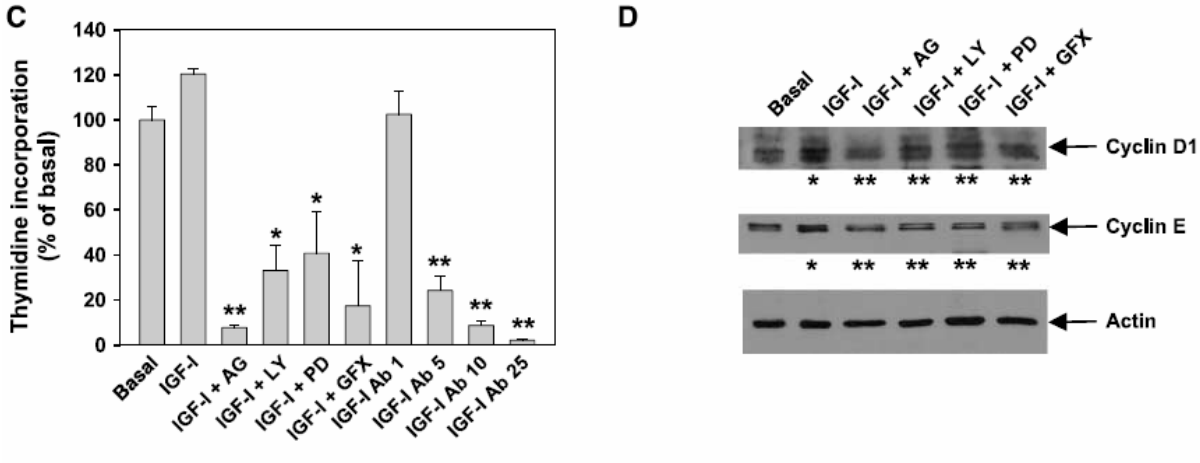


Figure 12 C, D. Effects of IGF-I and IGF-I pathway inhibitors on estradiol production and R2C cell proliferation. Cells were treated in serum-free medium for 24 h with IGF-I (100 ng/mL)alone or in combination with AG1024 (AG) (20 μmol/L), LY294002 (LY) (10 μmol/L), PD98059 (PD) (20 μmol/L), and GF109203X (GFX) (20 μmol/L). **(C)**, R2C cell proliferation was evaluated by [3H]thymidine incorporation analysis. Cells were maintained for 24 h in serum-free medium and treated for 24 h as indicated. IGF-I antibody (IGF-I Ab) was added to the medium at 1, 5, 10, and 25 μg/mL. *Columns*, mean percent of untreated (basal) cells (100%) from three independent experiments each done in triplicate; *bars*, SE. *, $P < 0.01$; **, $P < 0.001$, compared with IGF-I. **(D)**, Western blot analyses were done on 50 μg of total proteins extracted from R2C cells treated as indicated. Representative of three independent experiments with similar results. β-Actin was used as a loading control. Normalized absorbances were subjected to statistical analysis; statistically significant differences are indicated (*, $P < 0.01$, compared with basal; **, $P < 0.01$, compared with IGF-I).

COX-2 is highly expressed in tumor Leydig samples and is necessary for aromatase expression

Using western analysis we investigated COX-2 expression in normal and tumor Leydig samples (Fig. 13A). Both R2C cells in basal condition as well as testes from Fischer rats with a developed tumor (FRTT) express increased COX-2, that is absent in normal controls. Moreover, we found similar results in human samples; the cytoplasm of neoplastic human Leydig cells showed a strong COX2 immunoreactivity, while immunonegative nuclei displayed only the blue counterstaining (Fig. 13B). Control human testes showed immunonegative reaction for COX-2 (Fig 13B).

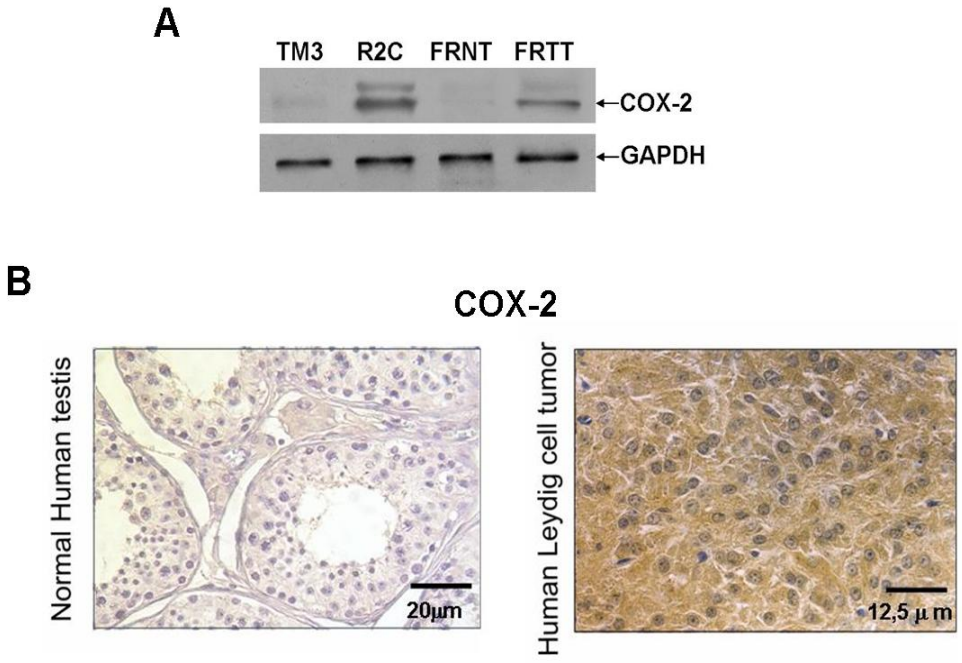


Figure 13. Expression of Cyclooxygenase-2 in tumor Leydig samples. (A) Western blot analysis was performed on 50 µg of total proteins extracted from TM3 and R2C cells or from total tissue of normal (FRNT) and tumor (FRTT) Fischer rat testes. GAPDH was used as a loading control. Results are representative of three independent experiments. **(B)** Immunolocalization of COX-2 in normal human testis and in testis with a Leydig cell tumor. Scale bars = 20µm (normal human testis); 12,5µm (human Leydig cell tumor). Brown (diaminobenzidine) cytoplasmic staining is indicative of COX-2 immunoreactivity.

Same experiments performed with COX-1 antibodies did not give different levels of expression between rat and human tumoral samples (data not shown). We have previously shown that expression of aromatase, SF-1 and phosphorylated form of CREB are higher in rat tumor Leydig cells. The altered activation of CREB, together with higher SF-1 expression, can explain the increase in aromatase levels observed in tumor Leydig cells. Knock down of COX-2 in R2C cells, using siRNA, caused a significant decrease in aromatase expression together with a decrease in CREB phosphorylation (Fig. 14).

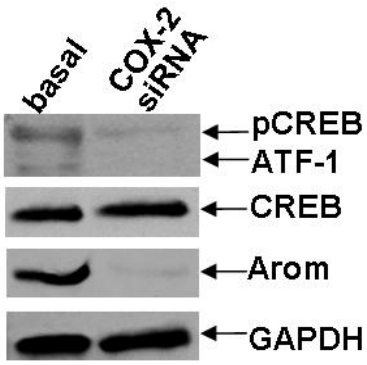


Figure 14. Effects of knocking down COX-2 on CREB and aromatase expression. R2C cells were transfected in serum-free medium with COX-2 (100nM) siRNA. After 48h western blot analysis of pCREB, CREB and aromatase were performed on 50 μg of total protein extracted from R2C cells transfected with scrambled siRNA (basal) or with COX-2 siRNA (COX-2 siRNA). Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control.

COX-2 inhibitor NS398 decreases pCREB and aromatase expression

To investigate whether COX-2 is involved in the mechanism determining pCREB activation in R2C cells we used a specific COX-2 inhibitor NS398 (NS). Western blot analyses showed that addition of increasing doses of NS (5, 25 and 50 μmol/L) caused a decrease in both phosphorylated CREB, as seen using a specific anti pCREB antibody, and aromatase levels (Fig. 15A). A drop in aromatase expression was also reflected by a change in enzymatic activity, that was dramatically reduced with all the investigated NS398 doses (Fig. 15B).

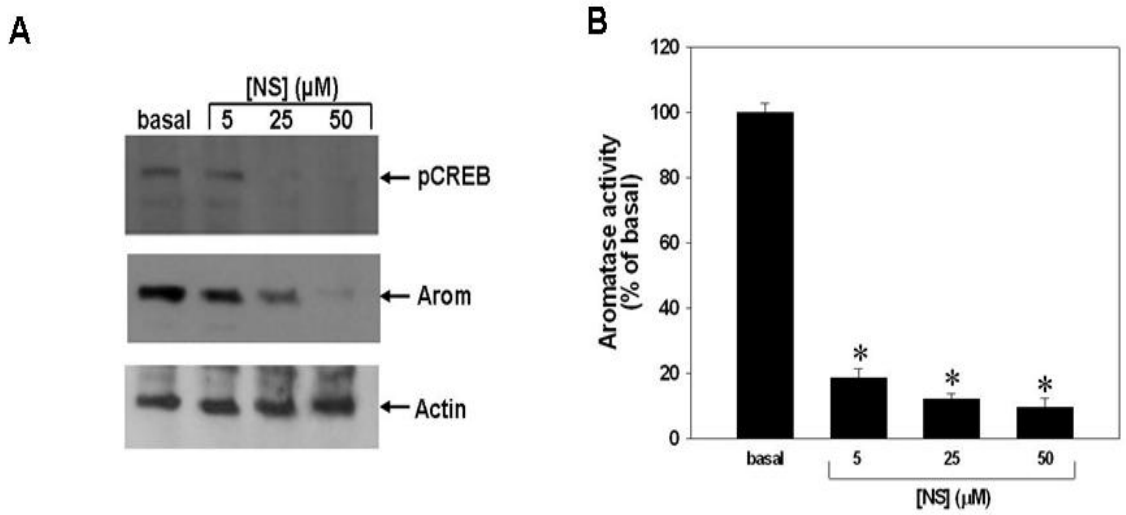


Figure 15 A, B. Effects of COX-2 inhibitor on aromatase expression in R2C cells. (A) Western blot analysis of pCREB and aromatase were performed on 50 μg of total proteins extracted from R2C cells non treated (basal) and treated with NS398 (5, 25, 50 μmol/L) for 24h. Blots are representative of three independent experiments with similar results. β-actin was used as a loading control. **(B)** R2C cells were untreated (basal) or treated for 24h with the increasing amounts of NS398 (5, 25, 50 μmol/L). Aromatase activity was assessed by using the modified tritiated water method. Results obtained were calculated as pmoles of [3H]H2O released per hour normalized to the well protein content (pmol/h/mg protein) and expressed as percent of basal. *Columns*, mean of three independent experiments each done in triplicate; *bars*, SE. *, $P < 0.01$, compared with basal.

Being active CREB a transcription factor necessary for aromatase transcription via PII promoter, these observation could potentially reflect changes on the mRNA levels. As seen by real time RT-PCR, addition of the different doses of NS398, determined a decrease in aromatase mRNA (Fig. 15C), without affecting CREB levels (Fig. 15D), indicating a specific effect of COX-2 inhibition on the phosphorylative protein status.

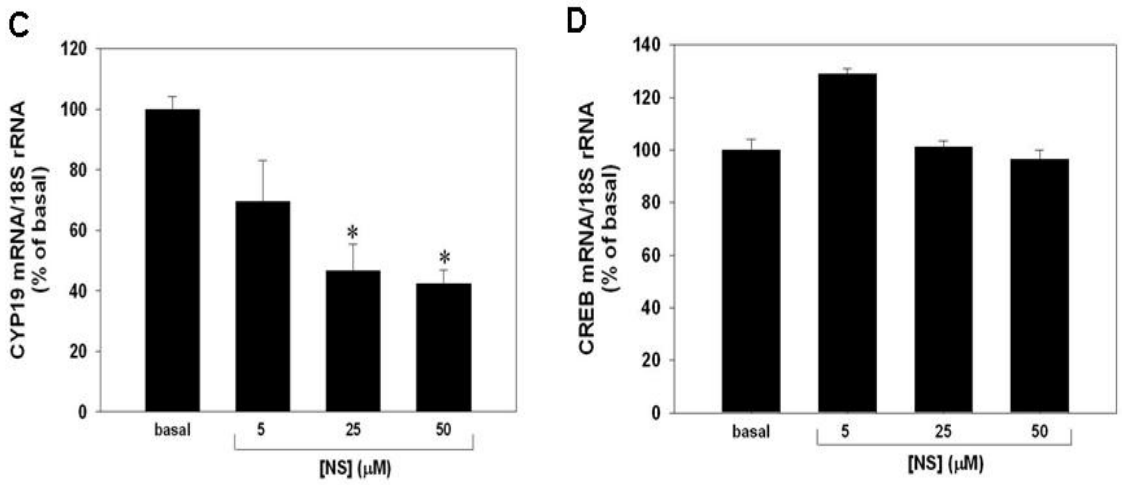


Figure 15 C, D. Effects of COX-2 inhibitor on aromatase expression in R2C cells. Total RNA was extracted from R2C cells untreated (basal) or treated for 24 h with the indicated doses of NS398. Real-time reverse transcription-PCR was used to analyze mRNA levels of CYP19 **(C)** and CREB **(D)**. *Columns*, mean of values from three separate RNA samples; *bars*, SE. Each sample was normalized to its 18S rRNA content. *, $P < 0.01$ compared with basal.

The effects of NS398 on aromatase expression led us to hypothesize that potentially, there could be a decrease in pCREB binding to the aromatase PII promoter responsible for a decreased gene transcription. With a ChIP assay, we were able to show a specific decrease in pCREB binding (Fig. 16A) without any change in the amount of total CREB protein present on the aromatase PII promoter (Fig. 16B).

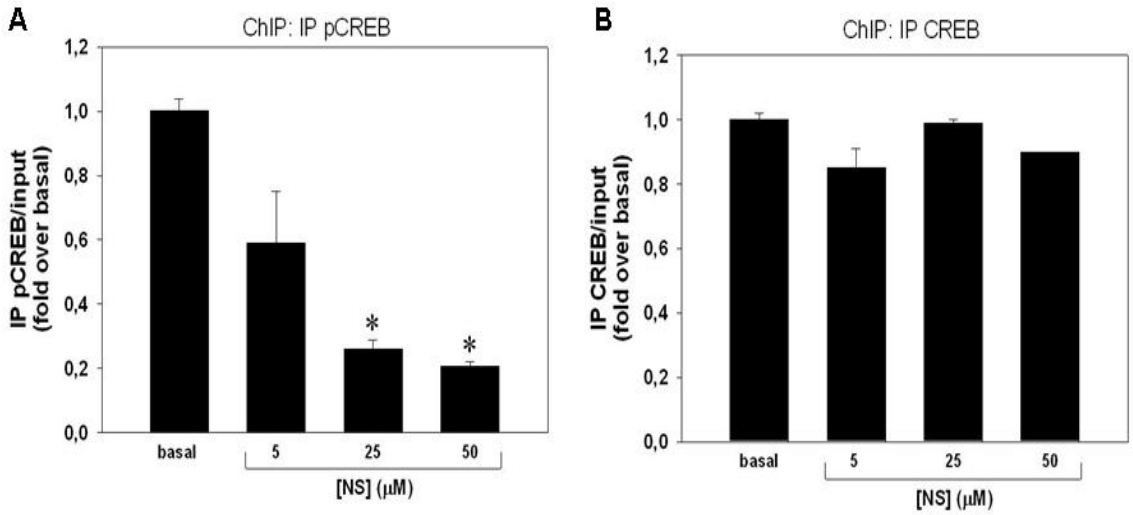


Figure 16. Effects of COX-2 inhibitor on pCREB and CREB binding to aromatase PII promoter in R2C cells. ChIP assays were performed on R2C cells untreated (basal) or treated for 24 h with the indicated doses of NS398. Immunoprecipitated (pCREB and CREB) and total (10% input) DNA were subject to real time PCR using specific primers **(A, B)**. Untreated cells (basal) were treated with the same amount of vehicle alone (DMSO) that never exceeded 0.01% (v/v). *Columns*, mean of values from three separate experiments; *bars*, SE. Each sample was normalized to the amplified input values. *, $P < 0.01$ compared with basal.

PGE2 activated pathway regulates aromatase expression

Either isoform of COX is responsible for prostaglandins (PGs) synthesis. Starting from previous findings demonstrating that PGE2 stimulates aromatase expression (267;276) we investigated the role of this factor in the regulation of aromatase in our cell model.

Addition of exogenous amounts of PGE2 to R2C cells were able to increase CREB phosphorylation and consequently aromatase expression (Fig. 17A). Since PGE2 works binding to four different receptor isoforms, named EP, we tested the effect of selective inhibitors (EP1 [SC19220], of EP2/EP4 [AH6809], of EP4 [AH23848]) on pCREB and aromatase levels (Fig. 17B). Among the tested antagonist only the one selective for the EP4, AH23848 was able to produce an effect; specifically, a decrease in pCREB and in aromatase levels.

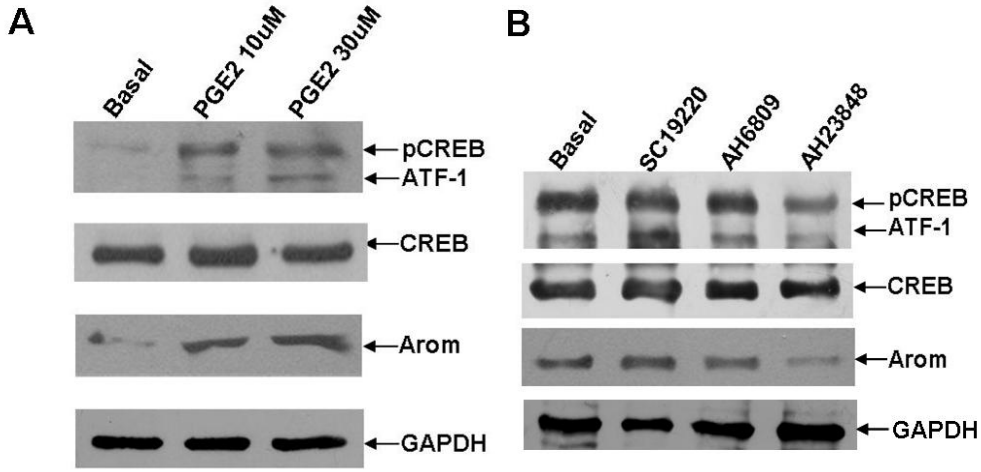


Figure 17. Effects of PGE2 administration and EP inhibitors on CREB and aromatase expression in R2C cells. (A) Western blot analysis of pCREB, CREB and aromatase were performed on 50 μg of total proteins extracted from R2C cells non treated (basal) and treated with PGE2 (10 and 30 μmol/L) for 48h. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control. **(B)** Western blot analysis of pCREB, CREB and aromatase were performed on 50 μg of total proteins extracted from R2C cells non treated (basal) and treated with EP inhibitors (SC19220, AH6809, AH23848) (10 μmol/L) for 48h. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control.

Moreover, addition of PGE2 abrogated the inhibition on aromatase activity caused by AH23848 (Fig 18).

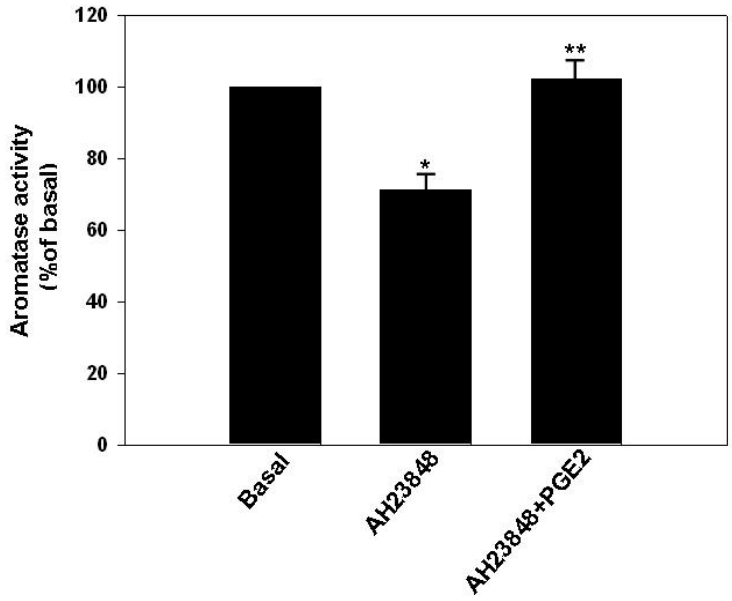


Figure 18. Effects of PGE2 and AH23848 on aromatase activity in R2C cells. Cells were cultured for 24h in serum-free HAM-F10 and then treated for 48 h with AH23848 (10 μmol/L). Medium was then removed and replaced with medium only or where indicated with PGE2 (30μmol/L). Aromatase activity was assessed by using the modified tritiated water method. Results obtained were calculated as pmoles of [3H]H2O released per hour normalized to the well protein content (pmol/h/mg protein) and expressed as percent of basal. *Columns*, mean of three independent experiments each done in triplicate; *bars*, SE. *, $P < 0.01$, compared with basal; **, $P < 0.01$, compared with AH23848.

PKA inhibitor H89 decreases aromatase expression and activity as a consequence of reduced pCREB activation

EP4 transduces its signal activating PKA, and we wanted to test the effect of a specific PKA inhibitor, H89, on aromatase expression. H89 decreased in a dose dependent manner both phosphorylated CREB and aromatase levels (Fig. 19A). A drop in aromatase expression was also reflected by a change in enzymatic activity, that was reduced with all the investigated H89 doses (Fig. 19B).

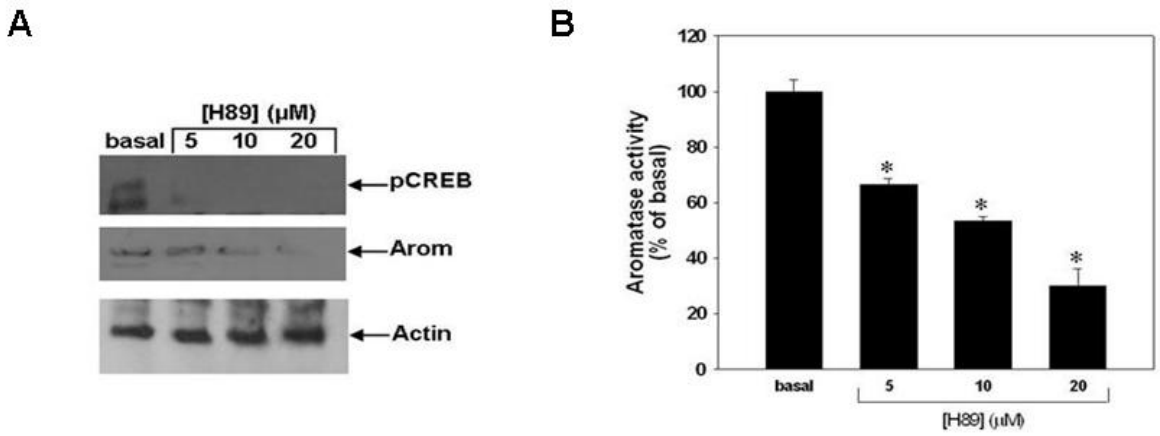


Figure 19 A, B. Effects of PKA inhibitor H89 on aromatase expression in R2C cells. (A) Western blot analysis of pCREB and aromatase were performed on 50 μg of total proteins extracted from R2C cells non treated (basal) and treated with H89 (5, 10, 20 μmol/L) for 24h. Blots are representative of three independent experiments with similar results. β-actin was used as a loading control. **(B)** R2C cells were untreated (basal) or treated for 24h with the increasing amounts of H89 (5, 10, 20 μmol/L). Aromatase activity was assessed by using the modified tritiated water method. Results obtained were calculated as pmoles of [3H]H2O released per hour normalized to the well protein content (pmol/h/mg protein) and expressed as percent of basal. *Columns*, mean of three independent experiments each done in triplicate; *bars*, SE. *, $P < 0.01$, compared with basal.

Similarly, H89 decreased aromatase mRNA (Fig.19C), but had no effect on CREB mRNA levels (Fig. 19D).

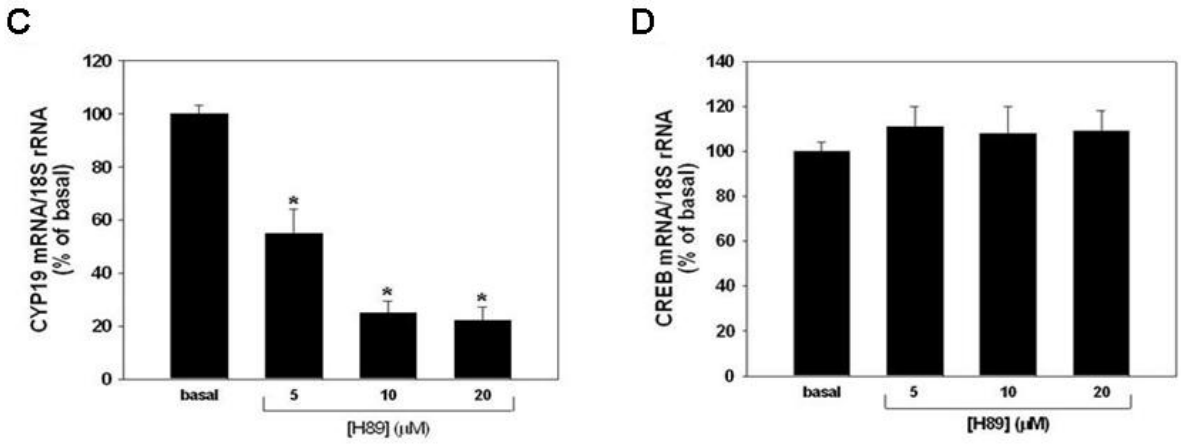
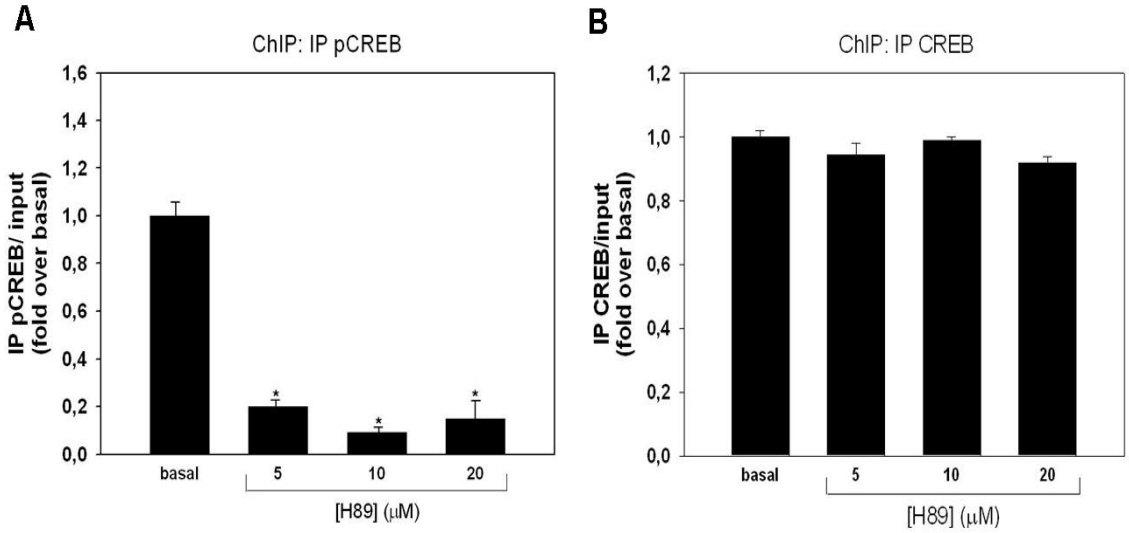


Figure 19 C, D. Effects of PKA inhibitor H89 on aromatase expression in R2C cells. Total RNA was extracted from R2C cells untreated (basal) or treated for 24 h with the indicated doses of H89. Real-time reverse transcription-PCR was used to analyze mRNA levels of CYP19 **(C)** and CREB **(D)**. *Columns*, mean of values from three separate RNA samples; *bars*, SE. Each sample was normalized to its 18S rRNA content. *, $P < 0.001$ compared with basal.

The decrease in aromatase expression was due to a decrease in pCREB binding to the aromatase PII promoter (Fig. 20A), while the amount of total CREB protein present on the aromatase PII promoter did not change (Fig. 20B).



F**igure 20. Effects of PKA inhibitor on CREB and pCREB binding to aromatase PII promoter in R2C cells.** ChIP assays were performed on R2C cells untreated (basals) or treated as indicated. Untreated cells (basal) were treated with the same amount of vehicle alone (DMSO) that never exceeded 0.01% (v/v). Immunoprecipitated (pCREB and CREB) and total (10% input) DNA were subject to real time PCR using specific primers **(A, B)**. *Columns*, mean of values from three separate experiments; *bars*, SE. Each sample was normalized to the amplified input values. *, $P < 0.01$ compared with basal.

A further demonstration of PKA implication in all the observed effects derives from the direct measurement of kinase activity after treatment with the inhibitors (Fig. 21). As expected H89 caused a 58% inhibition of PKA activity after 4h treatment, similarly NS398 and AH23848 determined 33 and 70 % inhibition respectively (Fig. 21).

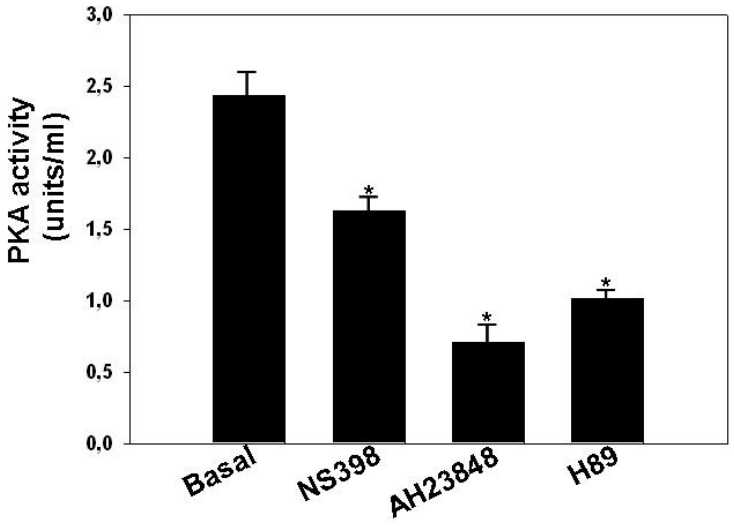


Figure 21. Effect of NS398, AH23848 and H89 on PKA activation. Cell lysates were analyzed for PKA activity by non-radioactive in vitro PKA assay as described in materials and methods. R2C cells were treated for 4h with NS398 (50 μmol/L), AH23848 (10 μmol/L) and H89 (20 μmol/L). Absorbance of phosphorylated peptide was quantified by spectrophotometric method. Results are mean ± S.D. of three independent experiments. *P <0.001 compared to basal.

Inhibition of PGE_2 dependent pathway decreases estradiol production and consequently tumor Leydig cell proliferation

The involvement of COX-2 through PGE2 production and PKA activation in controlling aromatase activity in tumor Leydig cell is further supported by the ability of NS398, AH23848 and H89 to inhibit basal estradiol production (Fig. 22).

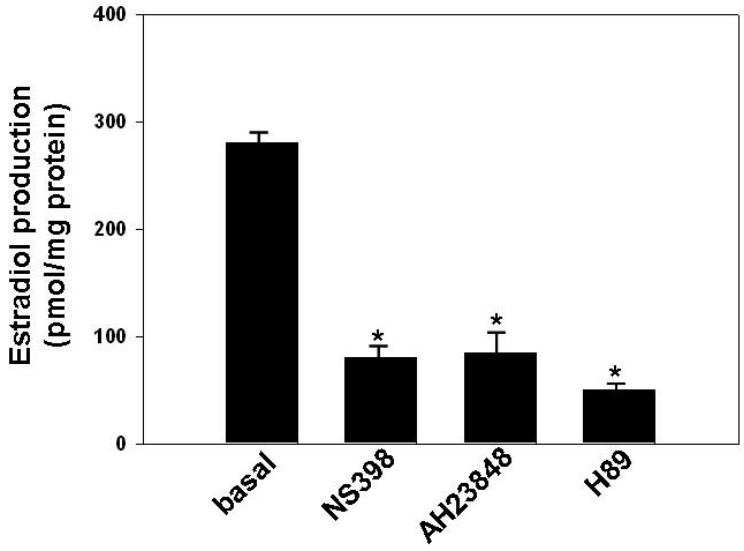


Figure 22. Effects of NS398, AH23848 and H89 on estradiol production. Cells were maintained for 24 h in serum free HAM-F10 medium before being treated for 48 h with NS398 (50 μmol/L), AH23848 (10μmol/L) or H89 (10 μmol/L) or maintained untreated (basal). E2 content in culture medium was determined by RIA and normalized to the cell culture well protein content. *Columns*, mean of three independent experiments each done in triplicate; *bars*, SE. *, $P < 0.05$, compared with basal.

As expected, the effect of decreased E2 production was a decrease in cell proliferation, that with the higher doses of both NS398, AH23848 or H89 was 72%, 70% and 82% respectively (Fig. 23 A, B, C).

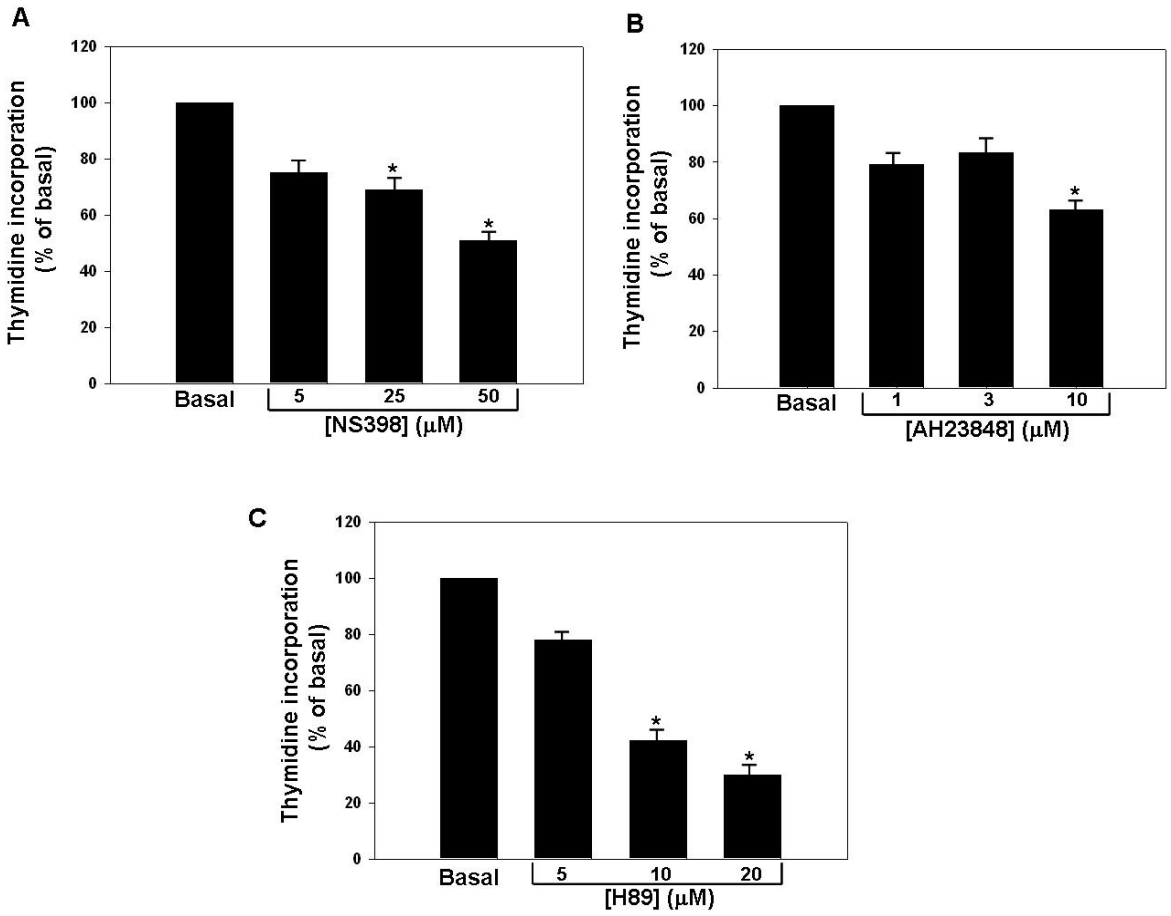


Figure 23. Effects of NS398, AH23848 and H89 on Leydig cell proliferation. (B, C) Cells were treated in serum-free medium in the absence (basal) or presence of NS398 **(A)**, AH23848 **(B)** or H89 **(C)** at the indicated concentrations for 72h after 24 h starvation. R2C cell proliferation was evaluated by [^{3}H] Thymidine incorporation analysis. Values expressed as percent of untreated (basal) cells (100%) represent the mean ± SEM of three independent experiments each performed in triplicate. (*) P< 0.05 compared with basal.

An additional demonstration of the involvement of COX-2 dependent pathway in controlling estradiol production in R2C cells derives from a evaluation of cell proliferation after knocking down COX-2 in these cells with a specific siRNA (Fig. 24). Thymidine incorporation was reduced by all the amounts of transfected COX-2 siRNA.

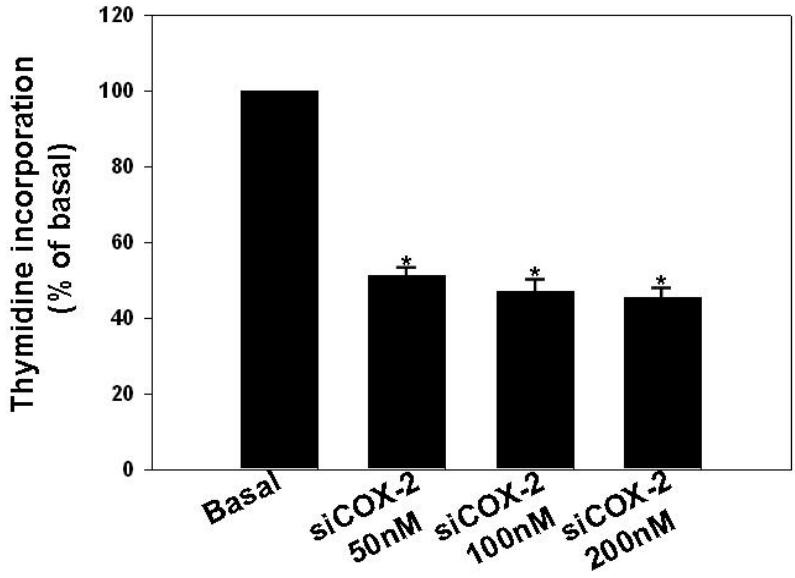


Figure 24. Effects of knocking down COX-2 on Leydig cell proliferation. R2C cells were transfected in serum-free medium with COX-2 siRNA at the indicated concentrations. After 48h cell proliferation was evaluated by [^{3}H] Thymidine incorporation analysis. Values expressed as percent of untreated (basal) cells (100%) represent the mean ± SEM of three independent experiments each performed in triplicate. (*) P< 0.05 compared with basal.

These changes in cell cycle can be explained by the decrease in Cyclin E expression determined by NS398 (Fig. 25A) , AH23848 (Fig. 25B) and H89 (Fig. 25C).

.

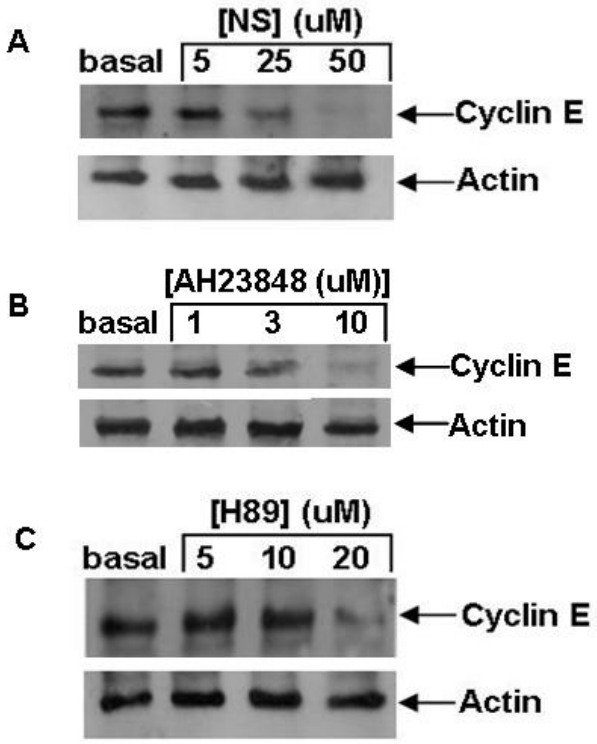


Figure 25. Effects of NS, AH23848 and H89 on cyclin E expression. Western blot analysis of cyclin E was performed on 50 μg of total protein extracted from R2C cells non treated (basal) and treated with NS398 (5, 25, 50 μmol/L), AH23848 (1, 3, 10 μmol/L) and H89 (5, 10, 20 μmol/L) for 24h. Blots are representative of three independent experiments with similar results. β-actin was used as a loading control.

These findings led us to suppose that in tumor Leydig cells the PGE2 derived from COX-2 overexpression could act through an autocrine mechanism in activating aromatase expression.

Discussion

The current study was aimed to explain the molecular mechanism responsible for aromatase overexpression in tumor Leydig cells with a consequent excess of estradiol *in situ* production sustaining tumor cell growth and proliferation.

Mammalian testis is capable of estrogen synthesis, whose production is regulated by different factors at different ages. In mature animals, aromatization of testosterone to estradiol is enhanced by LH/chorionic gonadotropin (CG) and not by FSH. The site of this synthesis appears to be age-dependent, at least in some species, such as the rat (285). Leydig cells are an elective target site of LH/CG which controls testosterone biosynthesis as well as its conversion to estradiol through aromatase activity. Leydig cell is also known to be the site of estrogen synthesis in several species, including mice (286), humans (287), suine (288), and sheep (289). Alterations in local estrogen synthesis may have significant consequences in malignancy of these cells. In the present study we observed that manteinance of R2C cells in the absence of serum induces a cospicous release of E2 from cellular storage in a time dependent manner. This synthesis was abrogated by treatment with Letrozole, an aromatase inhibitor, addressing how estrogen production is dependent on high constitutive aromatase activity.

A strongly increased aromatase expression was observed in R2C cells respect to the normal cell line control TM3 as well as in FRTT respect to FRNT. These findings concord with a previous study on human tissues showing that the increase in estrogen synthesis, as a consequence of a more intense aromatase activity, is higher in Leydig cell tumor fraction than in normal tissue surrounding the tumor of the same patient (290).

Mediators of the physiological effects of estrogens are the estrogen receptors (ER), α and β. ERα appears to be confined to Leydig cells in testicular tissue (58), while ERβ has been detected immunohistochemically in several rat testicular cell types, including Sertoli cells, germ cells, and peritubular cells (291). An enhanced expression of ERα resulting in an increased ERα /ERβ ratio was observed in R2C compared to TM3 cell line as well as in

FRTT respect to FRNT. This is in agreement with previous reports demonstrating that transgenic mice overexpressing aromatase have an enhanced occurrence of breast and Leydig cell tumors together with an enhanced expression of ERα in the tumoral tissue (139) . The latter findings address reasonably how an estrogen short autocrine loop may be involved in breast and testicular tumorogenesis in the presence of an excess of locally produced estradiol. Indeed, an arrest of cell growth was observed following abrogation of local E2 production with Letrozole or after addition of ERα inhibitors ICI or OHT. Besides, only after remotion of medium every day along with prolonged R2C starvation abolishing local steroid production, we observed how exogenous E2 was able to display proliferative effects.

One mechanism through which estrogens induce cell proliferation is by increasing protein levels of G1 regulatory cyclins A, B1, D1, D3, and E in target cells (292). In our study we showed that the expression of two of the most important regulators of Leydig cell cycle, cyclin D1 and E can be increased by E2 and downregulated by treatment with antiestrogens. These data further confirm that aromatase overexpression and the consequent E2 production may be the cause of altered cell cycle regulation of Leydig tumor cells.

In the attempt to explain the molecular mechanism determining aromatase overexpression in our tumor cell line, we focused our attention on expression levels of transcription factors identified as crucial regulators of aromatase gene expression: CREB and SF-1. In the adult testis SF-1 is predominantly expressed in Leydig cells (293). The increase of total and/or phosphorylated protein can potentiate SF-1 transcriptional activity (294). In R2C cells and in FRTT compared to the normal controls we found higher SF-1 phosphorylated protein levels as a consequence of elevated protein content. Total CREB levels were similar in all samples but highly phosphorylated in tumor samples. Starting from these observations we investigated which pathways could be involved in the activation of these transcription factors.

The most important signal that initiates steroidogenesis in Leydig cells is the binding of LH to the LH receptor (295). It has been demonstrated that LH/LHreceptor signaling

pathway is constitutively active in R2C cells and makes the phenotype of these cells constitutively steroidogenic (296). For instance in the presence of a specific PKA inhibitor, constitutive synthesis of Steroidogenic Acute Regulatory Protein (StAR) mRNA and steroids were significantly inhibited (297). These observations fit well with our findings evidencing how the presence of PKA inhibitor determined a strong decrease in aromatase activity together with a drop in CREB phosphorylation. In the presence of a specific PKC inhibitor no effects were elicited on phosphorylation of CREB, while SF-1 dropped dramatically.

It has been shown that CREB in mouse Leydig cells can be phosphorylated also through the PKC pathway, activated by IGF-I (296). In this study we have revealed that R2C tumor Leydig cells release higher levels of IGF-I in the culture medium respect to TM3 cells. However, the exposure to IGF-I as well as the treatment with inhibitors of IGF-I signalling did not affect CREB phosphorylative status but decreased SF-1 phosphorylation, postulating a separate mechanism controlling CREB and SF-1 activation in modulating aromatase activity.

These findings led us to suppose that the IGF-I derived from tumor Leydig cells could act through an autocrine mechanism in activating aromatase expression.

IGF-I receptors have previously been identified in Leydig cells of several species (188;198;298;299). It has been hypothesized that changes in IGF-RI expression can influence tumor cell progression. However in our cellular models, we did not reveal differences in IGF-RI expression between normal and tumor cells, indicating that IGF-I level may be the determining factor in potentiating IGF-I signalling. A previous study investigating the effects of long term IGF-I treatment on Leydig cells did not reveal alterations in DNA synthesis, indicating that IGF-I may act as a differentiation factor rather than a mitogenic factor (191). In fact, expression levels of all mRNA species associated with testosterone (T) biosynthesis were shown to be lower in the absence of IGF-I, while treatment with IGF-I/insulin has been found to stimulate steroidogenesis and StAR expression in Leydig cells through a process that does not require cAMP signalling (184;299;300).

In the same vein we may reasonable hypothesize that IGF-I could sustain, through an autocrine/paracrine mechanism, the elevated aromatase expression/activity in tumor Leydig cells. To verify this hypothesis we studied the various signalling pathways initiated by IGF-I through IGF-IR. Binding of IGF-I to its receptor causes receptor autophosphorylation and the activation of intrinsic tyrosine kinase that acts on various substrates including the insulin receptor substrate (IRS) and Shc adaptor proteins. These activated proteins recruit other factors, leading to activation of multiple signalling pathways including the phosphatidyl inositol 3-kinase (PI3K)/Akt and the mitogen-activated protein (MAP) kinase cascade. In addition, it has been shown that IGF-I can activate also the phospholipase C (PLC)/protein kinase C (PKC) pathway (3;301).

To demonstrate a role for IGF-I in mediating aromatase activation we used specific inhibitors for IGF-I signaling [AG1024 (AG)], ERK1/2 [PD98059 (PD)], PI3K [LY294002 (LY)] and PKC [GF109203X (GFX)] and showed a reduction of aromatase activity with all of them. Together these data confirm a role for IGF-I in mediating aromatase activation in tumor Leydig cells. All of the different inhibitors but PD were able to produce a similar inhibitory pattern on both aromatase and SF-1 mRNA and protein expression. Furthermore by ChIP assay we evidenced that SF-1 binding to the aromatase promoter II that was reduced by AG, LY, GFX but not by PD indicating a central role of this transcription factor in regulating aromatase gene transcription in tumor Leydig cells. This is the first report of a direct link between SF-1 transcription and IGF-I signalling pathway in regulating aromatase expression.

Furthermore, addition of IGF-I itself was able to increase aromatase activity and expression. These events were due to an increase in the amount of total and phosphorylated SF-1 levels whose binding to the aromatase promoter was shown to be rapidly augmented. So we postulate that an enhanced endogenous IGF-I local production may contribute to maintain an elevated aromatase activity sustained by a direct stimulatory effect of SF-1. For instance the inhibition of IGF-I signalling through inhibition of either PI3K/AKT and PLC/PKC pathways were able to block SF-1 expression and protein phoshorylation. Particularly treatment with AG blocked SF-1 phosphorylation more efficiently than the

separate treatment of PI3K or PKC, addressing how both pathways may synergize in upregulating SF-1 activity. In the presence of PD, SF-1 expression remained unchanged together with unaffected aromatase mRNA and protein levels. Importantly aromatase activity appeared decreased in the presence of PD suggesting a potential stimulatory role of ERK1/2 on the enzyme at a post-transcriptional level. From our findings then emerges a double mechanism inducing enhanced expression of aromatase: 1. a constitutive activation of PKA determining CREB phosphorylation; 2. an enhanced IGF-I signaling potentiating SF-1 action. The enhanced expression of SF-1 may be maintained by the lack of DAX-1 (Dosage-Sensitive Sex Reversal, Adrenal Hypoplasia Congenita, Critical Region on the X Chromosome, Gene-1) in R2C cells (283). DAX-1 is a specific co-repressor of SF-1 and inhibits StAR expression and steroidogenesis by 40-60% when overexpressed in R2C cells (283). The lack of DAX-1 expression in R2C cells may be due to the constitutive active PKA signalling, in fact since in a mouse Leydig cell line was shown a marked decrease of DAX-1 mRNA within 3 h after addition of LH or forskolin (302). Then, the activation of LH/LHr/PKA pathway decreases DAX-1 expession and promotes SF-1 activity, at the same time PKA activation if the cause an higher CREB phosphorylation and consequently activity. The mechanism responsible for PKA constitutive activation in tumor Leydig cells is yet to be defined.

Our hypothesis was that an increase in COX-2 levels and consequent prostaglandin synthesis could be the event responsible for PKA activation. This hypothesis was supported by immunostochemistry showing COX-2 overexpression in human Leydigioma samples and its absence in normal testes. In addition, western analysis for COX-2 on normal and tumor Leydig samples showed that COX-2 is highly expressed in R2C cells and in testicular lysates from Fischer with tumors.

Similarly, COX-2 is expressed in breast cancer (254) and it has been shown a correlation between aromatase and COX-2 expression in this type of tumor (259). Knock down of COX-2 in R2C cells using RNAi was able to decrease aromatase expression and to almost abolish CREB phosphorylation. These events produced by COX-2 knock down were also observed using a COX-2 selective antagonist, NS398. In addition, using ChIP assay, we

were able to show that the amount of pCREB bound to the PII promoter was significantly reduced, this implies that the cause of the observed reduction in aromatase expression could be due to a decrease in PKA-dependent CREB phosphorylation.

Several studies have been performed on aromatase regulation in breast cancer, which represents another estrogen-dependent type of tumor, meaning that it expresses estrogen receptor and requires estrogen for growth (303). Similarly to tumor Leydig cells, breast cancer cells have an increased aromatase expression (304), due to a switch in the promoter region utilized in gene expression. In breast cancer patients is found a switch from promote I.4 to promoter I.3 and II and I.7 (267;305-307) regulated through cAMP-mediated pathways. In addition, a previous study on adipose stromal cells surrounding breast cancer cells, showed PGE2 to be a potent activator of aromatase expression via promoter II. Furthermore, it was demonstrated that PGE2 is produced by breast tumors stimulating local estrogen biosynthesis in the surrounding adipose tissue, suggesting a potential paracrine/autocrine role for prostaglandins (PGs) in regulating aromatase expression in other cell types, including tumor Leydig cells.

Different prostaglandins are produced by the rat testis at different levels; in particular they include PGF2α (79,5%), PGE2 (20.3%) and PGD2 (0.17%) (308). PGE2-producing rat testicular cells include Leydig cells, Sertoli cells and spermatogenic cells and it has been implicated in the control of testicular steroidogenesis, spermatogenesis and local immunity (309). In addition, in the mouse there is also a correlation between COX-2 expression and PGE2 production in male genital organs (310). Even in another species, such as newt, it was observed that PGE2 increases 17β-estradiol, cAMP and aromatase activity while decreasing testosterone, probably as a consequence of increased conversion into estrogens (311). Consistent with findings related to COX-2 and tumor, PGE2 is also known to possess properties that promote malignant growth. For example, PGE2 stimulates angiogenesis, invasiveness and inhibits immune surveillance (312). PGs, produced through COX, exert their actions binding to specific transmembrane G-protein coupled receptors termed Prostanoid (P) receptors, present as different splice variants (270).

Specifically, PGE_2 receptors, EP_1(271) , EP_2(272), EP_3(273) and EP_4(274), are transduced through modulation of the activity of either adenylyl cyclase or inositol phospholipid hydrolysis and calcium mobilization (270;275). In particular, EP_1 activates PKC, EP_2 and EP_4 are coupled to PKA, EP_3 can either couple negatively to adenylyl cyclase through binding a G_i protein or associate to inositol phospholipid hydrolysis and calcium mobilization. Importantly, PGE_2 was shown to increase aromatase expression, while on the contrary EP_1 and EP_2 selective antagonists decreased the enzyme activity (276), suggesting that both PKA and PKC pathways are required for aromatase optimal expression.

In our experiments using selective antagonists for the different EP subtypes, we found that only AH23848, an EP4 inhibitor was able to decrease aromatase. EP4 transduces its signal through PKA, and in fact its block determines a decrease in CREB phosphorylation. These events are opposite to what observed using PGE2, which allows for increase in aromatase expression and in CREB phosphorylation.

R2C cells are known to have a constitutive active PKA activity (313), we measured kinase activity after treating R2C cells with COX-2 inhibitor NS398, EP4 inhibitor AH23848 and after treatment with a specific PKA inhibitor H89. All of the tested antagonists were able to decrease enzyme activity. The importance of PKA pathway in regulating aromatase expression in R2C cells can be seen in the experiments with H89, which specifically decreasing CREB phosphorylation determines a decrease in aromatase levels.

As is the case for IGF-I pathways inhibitors, that decrease estrogen production as a consequence of decreased aromatase expression, all of the tested inhibitors working at different levels on the same pathway, directly on either COX-2, EP4 or PKA determine a drop in the ability of R2C cells to produce estradiol. This event as previously shown causes a decrease in tumor cell proliferation, depending on the decrease of estrogen regulated cyclin such as cyclin E. The importance of COX-2/PGE2 dependent pathway is further confirmed by knocking down COX-2 expression with siRNA. The absence of this protein in R2C cells causes a decrease in tumor cell progression.

All these data complete our knowledge on the mechanisms involved in aromatase regulation in Leydig cell tumor: COX-2 over-expression inducing PGE2 sinthesis activates PKA, which increases CREB phosphorylation; phosphorylated CREB binds CYP19 PII promoter together with SF-1, regulated by IGF-I, and increase aromatase expression. Constitutive active aromatase produces higher estradiol levels which then, increase Leydig cell proliferation (Diagram 1).

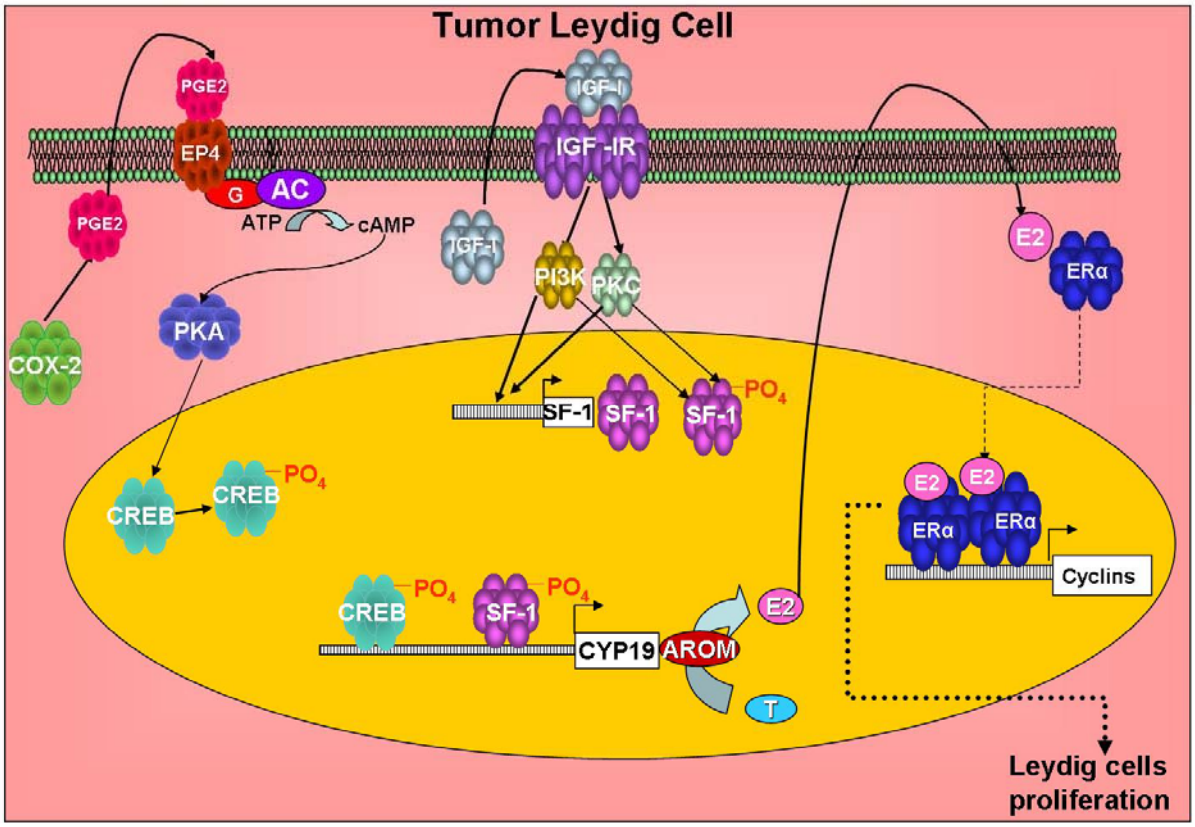


Diagram 1. Schematic model showing the mechanism of tumor Leydig cell proliferation.

Targeted inhibition of COX-2 and/or PGE2 are now regarded as poteintial strategies to stop completely the occurrence or progress of cancers. Selective COX-2 inhibitors are used for treatment of colorectal polyps in humans (243;314;315) women with high grade cervical dysplasia (316), and experimental studies showed that specific inhibition of COX-2 or PGE2 led to significant *in vivo* tumor reduction in murine lung cancer models (244).

In fact, the observation that antiestrogens and aromatase inhibitors, IGF-I signalling blockers as well as COX-2 inhibitor are able to reduce R2C proliferation opens new perspectives on the adjuvant therapeutic approach of testicular cancer. Remains to explain which molecular mechanism(s) is responsible for the elevated IGF-I production in tumor Leydig cells. In vivo, administration of hCG increases IGF-I mRNA levels in rat Leydig cells (317). LH deprival determines a decrease in BrdU incorporation as well as a decrease in mRNA levels of IGF-I and IGF-I receptor (318). These observations together with our data showing a decrease in IGF-I basal production after treatment with a PKA inhibitor (data not shown) suggest the possibility that LH can mediate its proliferative effects also by regulating IGF-I and its receptor in Leydig cells and that the altered LH/LHreceptor activated pathway in R2C cells could be the cause of IGF-I overproduction .

The mechanism detemining COX-2 expression and if the constitutive activation of LH/LHr/PKA signalling in R2C cells may be involved in upregulation of IGF-I expression remains also to be explored.

References

References

1. **McLachlan JA, Newbold RR, Bullock B** 1975 Reproductive tract lesions in male mice exposed prenatally to diethylstilbestrol. Science 190:991-992

2. **Newbold RR, Bullock BC, McLachlan JA** 1985 Lesions of the rete testis in mice exposed prenatally to diethylstilbestrol. Cancer Res 45:5145-5150

3. **Manna PR, Chandrala SP, King SR, Jo Y, Counis R, Huhtaniemi IT, Stocco DM** 2006 Molecular Mechanisms of Insulin-like Growth Factor-I Mediated Regulation of the Steroidogenic Acute Regulatory Protein in Mouse Leydig Cells. Mol Endocrinol 20:362-378

4. **De Kretser DM, Kerr JB** 1994 The cytology of the testis. ThePhysiology of Reproduction. E Knobil & JD Neill. New York: Raven Press ed.; 1177-1290

5. **Gaytan F, Bellido C, Aguilar E, van Rooijen N** 1994 Requirement for testicular macrophages in Leydig cell proliferation and differentiation during prepubertal development in rats. J Reprod Fertil 102:393-399

6. **Gaytan F, Bellido C, Morales C, Reymundo C, Aguilar E, van Rooijen N** 1994 Effects of macrophage depletion at different times after treatment with ethylene dimethane sulfonate (EDS) on the regeneration of Leydig cells in the adult rat. J Androl 15:558-564

7. **Borland K, Mita M, Oppenheimer CL, Blinderman LA, Massague J, Hall PF, Czech MP** 1984 The actions of insulin-like growth factors I and II on cultured Sertoli cells. Endocrinology 114:240-246

8. **De Kretser DM, Catt KJ, Paulsen CA** 1971 Studies on the in vitro testicular binding of iodinated luteinizing hormone in rats. Endocrinology 88:332-337

9. **Loosfelt H, Misrahi M, Atger M, Salesse R, Vu Hai-Luu Thi M, Jolivet A, Guiochon-Mantel A, Sar S, Jallal B, Garnier J, al. e** 1989 Cloning and sequencing of porcine LH-hCG receptor cDNA: variants lacking transmembrane domain. Science 245:525-528

10. **Dufau ML** 1988 Endocrine Regulation and Communicating Functions of the Leydig Cell. Annual Review of Physiology 50:483-508

11. **Cooke BA** 1990 Is cyclic AMP an obligatory second messenger for luteinizing hormone? Molecular and Cellular Endocrinology 69:C11-C15

12. **Walker WH, Cheng J** 2005 FSH and testosterone signaling in Sertoli cells. Reproduction 130:15-28

13. **Baird DT, Galbraith A, Fraser IS, Newsam JE** 1973 The concentration of oestrone and oestradiol-17 in spermatic venous blood in man. J Endocrinol 57:285-288

14. **Freeman DA, Ascoli M** 1982 Studies on the source of cholesterol used for steroid biosynthesis in cultured Leydig tumor cells. J Biol Chem 257:14231-14238

15. **Chung BC, Matteson KJ, Voutilainen R, Mohandas TK, Miller WL** 1986 Human Cholesterol Side-Chain Cleavage Enzyme, P450scc: cDNA Cloning, Assignment of the Gene to Chromosome 15, and Expression in the Placenta. PNAS 83:8962-8966

16. **Pollack SE, Furth EE, Kallen CB, Arakane F, Kiriakidou M, Kozarsky KF, Strauss JF, III** 1997 Localization of the Steroidogenic Acute Regulatory Protein in Human Tissues. Journal of Clinical Endocrinology Metabolism 82:4243-4251

17. **Stocco DM, Clark BJ** 1996 Regulation of the acute production of steroids in steroidogenic cells. Endocr Rev 17:221-244

18. **Clark BJ, Wells J, King SR, Stocco DM** 1994 The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). J Biol Chem 269:28314-28322

19. **Lin D, Sugawara T, Strauss JF, III, Clark BJ, Stocco DM, Saenger P, Rogol A, Miller WL** 1995 Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. Science 267:1828-1831

20. **Caron KM, Soo SC, Wetsel WC, Stocco DM, Clark BJ, Parker KL** 1997 Targeted disruption of the mouse gene encoding steroidogenic acute regulatory protein provides insights into congenital lipoid adrenaláhyperplasia. PNAS 94:11540-11545

21. **Weusten JJ, Smals AG, Hofman JA, Kloppenborg PW, Benraad TJ** 1987 Early time sequence in pregnenolone metabolism to testosterone in homogenates of human and rat testis. Endocrinology 120:1909-1913

22. **Matteson KJ, Picado-Leonard J, Chung BC, Mohandas TK, Miller WL** 1986 Assignment of the gene for adrenal P450c17 (steroid 17 alpha-hydroxylase/17,20 lyase) to human chromosome 10. J Clin Endocrinol Metab 63:789-791

23. **Chung BC, Picado-Leonard J, Haniu M, Bienkowski M, Hall PF, Shively JE, Miller WL** 1987 Cytochrome P450c17 (Steroid 17alpha -hydroxylase/17,20 Lyase): Cloning of Human Adrenal and Testis cDNAs Indicates the Same Gene is Expressed in Both Tissues. PNAS 84:407-411

24. **Inano H, Ishii-Ohba H, Sugimoto Y, Ohta Y, Morikawa T, Yoshida M, Tamaoki B** 1990 **Purification and properties of enzymes related to steroid hormone synthesis**. Ann N Y Acad Sci 595:17-25

25. **Luu-The V, Labrie C, Simard J, Lachance Y, Zhao HF, Couet J, Leblanc G, Labrie F** 1990 Structure of two in tandem human 17 beta-hydroxysteroid dehydrogenase genes. Mol Endocrinol 4:268-275

26. **Lorence MC, Corbin CJ, Kamimura N, Mahendroo MS, Mason JI** 1990 Structural analysis of the gene encoding human 3 beta-hydroxysteroid dehydrogenase/delta 5----4-isomerase. Mol Endocrinol 4:1850-1855

27. **Andersson S, Russell DW** 1990 Structural and Biochemical Properties of Cloned and Expressed Human and Rat Steroid 5 {alpha}-Reductases. PNAS 87:3640-3644

28. **Chen SA, Besman MJ, Sparkes RS, Zollman S, Klisak I, Mohandas T, Hall PF, Shively JE** 1988 Human aromatase: cDNA cloning, Southern blot analysis, and assignment of the gene to chromosome 15. DNA 7:27-38

29. **Akingbemi B** 2005 Estrogen regulation of testicular function. Reproductive Biology and Endocrinology 3:51-63

30. **Carreau S, Silandre D, Bourguiba S, Hamden K, Said L, Lambard S, Galeraud-Denis I, Delalande C.** 2007 Estrogens and male reproduction: a new concept. Braz J Med Biol Res 40:761-768

31. **Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Lorence S, Amarneh B, Ito Y, Fisher CR, Michael MD** 1994 Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr Rev 15:342-355

32. **Sebastian S, Bulun SE** 2001 A Highly Complex Organization of the Regulatory Region of the Human CYP19 (Aromatase) Gene Revealed by the Human Genome Project. Journal of Clinical Endocrinology Metabolism 86:4600-4602

33. **Simpson ER, Clyne C, Rubin G, Boon WC, Robertson K, Britt K, Speed C, Jones M** 2002 Aromatase--a brief overview. Annu Rev Physiol 64:93-127

34. **Graddy LG, Kowalski AA, Simmen FA, Davis SLF, Baumgartner WW, Simmen RCM** 2000 Multiple isoforms of porcine aromatase are encoded by three distinct genes. The Journal of Steroid Biochemistry and Molecular Biology 73:49-57

35. **Kishida M, Callard GV** 2001 Distinct Cytochrome P450 Aromatase Isoforms in Zebrafish (Danio rerio) Brain and Ovary Are Differentially Programmed and Estrogen Regulated during Early Development. Endocrinology 142:740-750

36. **Conley A, Hinshelwood M** 2001 Mammalian aromatases. Reproduction 121:685-695

37. **Means GD, Kilgore MW, Mahendroo MS, Mendelson CR, Simpson ER** 1991 Tissue-specific promoters regulate aromatase cytochrome P450 gene expression in human ovary and fetal tissues. Mol Endocrinol 5:2005-2013

38. **Birk OS, Casiano DE, Wassif CA, Cogliati T, Zhao L, Zhao Y, Grinberg A, Huang S, Kreidberg JA, Parker KL, Porter FD, Westphal H** 2000 The LIM homeobox gene Lhx9 is essential for mouse gonad formation. Nature 403:909-913

39. **Hickey GJ, Krasnow JS, Beattie WG, Richards JS** 1990 Aromatase cytochrome P450 in rat ovarian granulosa cells before and after luteinization: adenosine 3',5'-monophosphate-dependent and independent regulation. Cloning and sequencing of rat aromatase cDNA and 5' genomic DNA. Mol Endocrinol 4:3-12

40. **Young M, McPhaul MJ** 1998 A Steroidogenic Factor-1-Binding Site and Cyclic Adenosine 3',5'-Monophosphate Response Element-Like Elements Are Required for the Activity of the Rat Aromatase Promoter in Rat Leydig Tumor Cell Lines. Endocrinology 139:5082-5093

41. **Wang ZJ, Jeffs B, Ito M, Achermann JC, Yu RN, Hales DB, Jameson JL** 2001 Aromatase (Cyp19) expression is up-regulated by targeted disruption of Dax1. PNAS 98:7988-7993

42. **Pezzi V, Sirianni R, Chimento A, Maggiolini M, Bourguiba S, Delalande C, Carreau S, Ando S, Simpson ER, Clyne CD** 2004 Differential Expression of Steroidogenic Factor-1/Adrenal 4 Binding Protein and Liver Receptor Homolog-1 (LRH-1)/Fetoprotein Transcription Factor in the Rat Testis: LRH-1 as a Potential Regulator of Testicular Aromatase Expression. Endocrinology 145:2186-2196

43. **Silandre D, Delalande C, Durand P, Carreau S** 2007 Three promoters PII, PI.f, and PI.tr direct the expression of aromatase (cyp19) gene in male rat germ cells. J Mol Endocrinol 39:169-181

44. **Teixeira CV, Silandre D, de Souza Santos AM, Delalande C, Sampaio FJ, Carreau S, da Fonte Ramos C** 2007 Effects of maternal undernutrition during lactation on aromatase, estrogen, and androgen receptors expression in rat testis at weaning. J Endocrinol 192:301-311

45. **Gruber CJ, Tschugguel W, Schneeberger C, Huber JC** 2002 Production and Actions of Estrogens. N Engl J Med 346:340-352

46. **Kraus WL, McInerney EM, Katzenellenbogen BS** 1995 Ligand-Dependent, Transcriptionally Productive Association of the Amino- and Carboxyl-Terminal Regions of a Steroid Hormone Nuclear Receptor. PNAS 92:12314-12318

47. **McInerney EM, Tsai MJ, O'Malley BW, Katzenellenbogen BS** 1996 Analysis of estrogen receptor transcriptional enhancement by a nuclear hormone receptorácoactivator. PNAS 93:10069-10073

48. **Giguere V** 1999 Orphan Nuclear Receptors: From Gene to Function. Endocr Rev 20:689-725

49. **Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, Gustafsson JA** 2001 Mechanisms of Estrogen Action. Physiol Rev 81:1535-1565

50. **Chang WY, Prins GS** 1999 Estrogen receptor-beta: implications for the prostate gland. Prostate 40:115-124

51. **Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA** 1997 Comparison of the Ligand Binding Specificity and Transcript Tissue Distribution of Estrogen Receptors {alpha} and {beta}. Endocrinology 138:863-870

52. **Kuiper GGJM, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA** 1998 Interaction of Estrogenic Chemicals and Phytoestrogens with Estrogen Receptor {beta}. Endocrinology 139:4252-4263

53. **Hall JM, McDonnell DP** 1999 The Estrogen Receptor {beta}-Isoform (ER{beta}) of the Human Estrogen Receptor Modulates ER{alpha} Transcriptional Activity and Is a Key Regulator of the Cellular Response to Estrogens and Antiestrogens. Endocrinology 140:5566-5578

54. **Hewitt SC, Korach KS** 2002 Estrogen receptors: structure, mechanisms and function. Rev Endocr Metab Disord 3:193-200

55. **Loomis AK, Thomas P** 2000 Effects of Estrogens and Xenoestrogens on Androgen Production by Atlantic Croaker Testes In Vitro: Evidence for a Nongenomic Action Mediated by an Estrogen Membrane Receptor. Biol Reprod 62:995-1004

56. **Razandi M, Pedram A, Merchenthaler I, Greene GL, Levin ER** 2004 Plasma Membrane Estrogen Receptors Exist and Functions as Dimers. Mol Endocrinol 18:2854-2865

57. **Bjornstrom L, Sjoberg M** 2005 Mechanisms of Estrogen Receptor Signaling: Convergence of Genomic and Nongenomic Actions on Target Genes. Mol Endocrinol 19:833-842

58. **O'Donnell L, Robertson KM, Jones ME, Simpson ER** 2001 Estrogen and Spermatogenesis. Endocr Rev 22:289-318

59. **Luconi M, Forti G, Baldi E** 2002 Genomic and nongenomic effects of estrogens: molecular mechanisms of action and clinical implications for male reproduction. The Journal of Steroid Biochemistry and Molecular Biology 80:369-381

60. **Carreau S, Bourguiba S, Lambard S, Galeraud-Denis I, Genissel C, Levallet J** 2002 Reproductive system: aromatase and estrogens. Molecular and Cellular Endocrinology 193:137-143

61. **Carreau S, Lambard S, Delalande C, is-Galeraud I, Bilinska B, Bourguiba S** 2003 Aromatase expression and role of estrogens in male gonad : a review. Reproductive Biology and Endocrinology 1:35

62. **Cooke PS, Young P, Hess RA, Cunha GR** 1991 Estrogen receptor expression in developing epididymis, efferent ductules, and other male reproductive organs. Endocrinology 128:2874-2879

63. **Rochira V, Balestrieri A, Madeo B, Baraldi E, Faustini-Fustini M, Granata ARM, Carani C** 2001 Congenital estrogen deficiency: in search of the estrogen role in human male reproduction. Molecular and Cellular Endocrinology 178:107-115

64. **Fisher JS, Millar MR, Majdic G, Saunders PT, Fraser HM, Sharpe RM** 1997 Immunolocalization of oestrogen receptor-a within the testis and excurrent ducts of the rat and marmoset monkey from perinatal life to adulthood. J Endocrinol 153:485-495

65. **Hess RA, Bunick D, Lee KH, Bahr J, Taylor JA, Korach KS, Lubahn DB** 1997 A role for oestrogens in the male reproductive system. Nature 390:509-512

66. **van Pelt AMM, de Rooij DG, van der Burg B, van der Saag PT, Gustafsson JA, Kuiper GGJM** 1999 Ontogeny of Estrogen Receptor-{beta} Expression in Rat Testis. Endocrinology 140:478-483

67. **Pelletier G, Labrie C, Labrie F** 2000 Localization of oestrogen receptor alpha, oestrogen receptor beta and androgen receptors in the rat reproductive organs. J Endocrinol 165:359-370

68. **Shughrue PJ, Lane MV, Scrimo PJ, Merchenthaler I** 1998 Comparative distribution of estrogen receptor-[alpha] (ER-[alpha]) and [beta] (ER-[beta]) mRNA in the rat pituitary, gonad, and reproductive tract. Steroids 63:498-504

69. **Couse JF, Korach KS** 1999 Estrogen Receptor Null Mice: What Have We Learned and Where Will They Lead Us? Endocr Rev 20:358-417

70. **Levallet J, Bilinska B, Mittre H, Genissel C, Fresnel J, Carreau S** 1998 Expression and immunolocalization of functional cytochrome P450 aromatase in mature rat testicular cells. Biol Reprod 58:919-926

71. **Carreau S, Bourguiba S, Lambard S, Galeraud-Denis I, Genissel C, Bilinska B, Benahmed M, Levallet J** 2001 Aromatase expression in male germ cells. The Journal of Steroid Biochemistry and Molecular Biology 79:203-208

72. **Hess R** 2003 Estrogen in the adult male reproductive tract: A review. Reproductive Biology and Endocrinology 1:52

73. **Nitta H, Bunick D, Hess RA, Janulis L, Newton SC, Millette CF, Osawa Y, Shizuta Y, Toda K, Bahr JM** 1993 Germ cells of the mouse testis express P450 aromatase. Endocrinology 132:1396-1401

74. **Janulis L, Bahr JM, Hess RA, Janssen S, Osawa Y, Bunick D** 1998 Rat testicular germ cells and epididymal sperm contain active P450 aromatase. Journal of Andrology 19:65-71

75. **Takeyama J, Suzuki T, Inoue S, Kaneko C, Nagura H, Harada N, Sasano H** 2001 Expression and Cellular Localization of Estrogen Receptors {{alpha}} and {beta} in the Human Fetus. Journal of Clinical Endocrinology Metabolism 86:2258-2262

76. **Pelletier G, El-Alfy M** 2000 Immunocytochemical Localization of Estrogen Receptors {{alpha}} and {beta} in the Human Reproductive Organs. Journal of Clinical Endocrinology Metabolism 85:4835-4840

77. **Kolasa A, Wiszniewska B, Marchlewicz M, Wenda-Rózewicka L** 2003 Localisation of oestrogen receptors (ERalpha and ERbeta) in the human and rat epididymides. Folia Morphol (Warsz) 62:467-469

78. **Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjold M, Gustafsson JA** 1997 Human Estrogen Receptor {beta}-Gene Structure, Chromosomal Localization, and Expression Pattern. Journal of Clinical Endocrinology Metabolism 82:4258-4265

79. **Pentikäinen V, Erkkilä K, Suomalainen L, Parvinen M, Dunkel L** 2000 Estradiol Acts as a Germ Cell Survival Factor in the Human Testis in Vitro. Journal of Clinical Endocrinology Metabolism 85:2057-2067

80. **Lambard S, Galeraud-Denis I, Saunders PT, Carreau S** 2004 Human immature germ cells and ejaculated spermatozoa contain aromatase and oestrogen receptors. J Mol Endocrinol 32:279-289

81. **Saunders PTK, Sharpe RM, Williams K, Macpherson S, Urquart H, Irvine DS, Millar MR** 2001 Differential expression of oestrogen receptor {{alpha}} and {beta} proteins in the testes and male reproductive system of human and non-human primates. Mol Hum Reprod 7:227-236

82. **Makinen S, Makela S, Weihua Z, Warner M, Rosenlund B, Salmi S, Hovatta O, Gustafsson JA** 2001 Localization of oestrogen receptors alpha and beta in human testis. Mol Hum Reprod 7:497-503

83. **Saunders PTK, Millar MR, Macpherson S, Irvine DS, Groome NP, Evans LR, Sharpe RM, Scobie GA** 2002 ER{beta}1 and the ER{beta}2 Splice Variant (ER{beta}cx/{beta}2) Are Expressed in Distinct Cell Populations in the Adult Human Testis. Journal of Clinical Endocrinology Metabolism 87:2706-2715

84. **Durkee TJ, Mueller M, Zinaman M.** 1998 Identification of estrogen receptor protein and messenger ribonucleic acid in human spermatozoa. Am J Obstet Gynecol 178:1288-1297

85. **Luconi M, Muratori M, Forti G, Baldi E** 1999 Identification and Characterization of a Novel Functional Estrogen Receptor on Human Sperm Membrane That Interferes with Progesterone Effects. Journal of Clinical Endocrinology Metabolism 84:1670-1678

86. **Lambard S, Galeraud-Denis I, Bouraïma H, Bourguiba S, Chocat A, Carreau S** 2003 Expression of aromatase in human ejaculated spermatozoa: a putative marker of motility. Mol Hum Reprod 9:117-124

87. **Aquila S, Sisci D, Gentile M, Middea E, Siciliano L, Ando S** 2002 Human Ejaculated Spermatozoa Contain Active P450 Aromatase. Journal of Clinical Endocrinology Metabolism 87:3385-3390

88. **Eddy EM, Washburn TF, Bunch DO, Goulding EH, Gladen BC, Lubahn DB, Korach KS** 1996 Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. Endocrinology 137:4796-4805

89. **Hess RA, Gist DH, Bunick D, Lubahn DB, Farrell A, Bahr J, Cooke PS, Greene GL** 1997 Estrogen receptor (alpha and beta) expression in the excurrent ducts of the adult male rat reproductive tract. J Androl 18:602-611

90. **Zhou Q, Clarke L, Nie R, Carnes K, Lai LW, Lien YH, Verkman A, Lubahn D, Fisher JS, Katzenellenbogen BS, Hess RA** 2001 Estrogen action and male fertility: Roles of the sodium/hydrogen exchanger-3 and fluid reabsorption in reproductive tract function. PNAS 98:14132-14137

91. **Lee KH, Finnigan-Bunick C, Bahr J, Bunick D** 2001 Estrogen Regulation of Ion Transporter Messenger RNA Levels in Mouse Efferent Ductules Are Mediated Differentially Through Estrogen Receptor (ER) {alpha} and ER{beta}. Biol Reprod 65:1534-1541

92. **Lee KH, Hess RA, Bahr JM, Lubahn DB, Taylor J, Bunick D** 2000 Estrogen Receptor {alpha} Has a Functional Role in the Mouse Rete Testis and Efferent Ductules. Biol Reprod 63:1873-1880

93. **Fisher CR, Graves KH, Parlow AF, Simpson ER** 1998 Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. PNAS 95:6965-6970

94. **Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA, Smithies O** 1998 Generation and reproductive phenotypes of mice lacking estrogen receptor beta. PNAS 95:15677-15682

95. **Robertson KM, O'Donnell L, Jones MEE, Meachem SJ, Boon WC, Fisher CR, Graves KH, McLachlan RI, Simpson ER** 1999 Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene. PNAS 96:7986-7991

96. **Robertson KM, O'Donnell L, Simpson ER, Jones MEE** 2002 The Phenotype of the Aromatase Knockout Mouse Reveals Dietary Phytoestrogens Impact Significantly on Testis Function. Endocrinology 143:2913-2921

97. **Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS** 1994 Estrogen Resistance Caused by a Mutation in the Estrogen-Receptor Gene in a Man. N Engl J Med 331:1056-1061

98. **Maffei L, Murata Y, Rochira V, Tubert G, Aranda C, Vazquez M, Clyne CD, Davis S, Simpson ER, Carani C** 2004 Dysmetabolic Syndrome in a Man with a Novel Mutation of the Aromatase Gene: Effects of Testosterone, Alendronate, and Estradiol Treatment. Journal of Clinical Endocrinology Metabolism 89:61-70

99. **Carani C, Granata ARM, Rochira V, Caffagni G, Aranda C, Antunez P, Maffei LE** 2005 Sex steroids and sexual desire in a man with a novel mutation of aromatase gene and hypogonadism. Psychoneuroendocrinology 30:413-417

100. **Morishima A, Grumbach MM, Simpson ER, Fisher C, Qin K** 1995 Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. Journal of Clinical Endocrinology Metabolism 80:3689-3698

101. **Herrmann BL, Saller B, Janssen OE, Gocke P, Bockisch A, Sperling H, Mann K, Broecker M** 2002 Impact of Estrogen Replacement Therapy in a Male with Congenital Aromatase Deficiency Caused by a Novel Mutation in the CYP19 Gene. Journal of Clinical Endocrinology Metabolism 87:5476-5484

102. **Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J, Korach KS, Simpson ER** 1997 Effect of testosterone and estradiol in a man with aromatase deficiency. N Engl J Med 337:91-95

103. **Raman JD, Schlegel PN** 2002 Aromatase inhibitors for male infertility. J Urol 167:624-629

104. **Visser JA, McLuskey A, Verhoef-Post M, Kramer P, Grootegoed JA, Themmen APN** 1998 Effect of Prenatal Exposure to Diethylstilbestrol on Mullerian Duct Development in Fetal Male Mice. Endocrinology 139:4244-4251

105. **Atanassova N, McKinnell C, Turner KJ, Walker M, Fisher JS, Morley M, Millar MR, Groome NP, Sharpe RM** 2000 Comparative Effects of Neonatal Exposure of Male Rats to Potent and Weak (Environmental) Estrogens on Spermatogenesis at Puberty and the Relationship to Adult Testis Size and Fertility: Evidence for Stimulatory Effects of Low Estrogen Levels. Endocrinology 141:3898-3907

106. **Sharpe RM, Atanassova N, McKinnell C, Parte P, Turner KJ, Fisher JS, Kerr JB, Groome NP, Macpherson S, Millar MR, Saunders PT** 1998 Abnormalities in functional development of the Sertoli cells in rats treated neonatally with

diethylstilbestrol: a possible role for estrogens in Sertoli cell development. Biol Reprod 59:1084-1094

107. **Li X, Nokkala E, Yan W, Streng T, Saarinen N, Warri A, Huhtaniemi I, Santti R, Makela S, Poutanen M** 2001 Altered Structure and Function of Reproductive Organs in Transgenic Male Mice Overexpressing Human Aromatase. Endocrinology 142:2435-2442

108. **Streng T, Li X, Lehtoranta M, Makela S, Poutanen M, Talo A, Tekmal RR, Santti R** 2002 Infravesical obstruction in aromatase over expressing transgenic male mice with increased ratio of serum estrogen-to-androgen concentration. J Urol 168:298-302

109. **Toppari J, Skakkebaek NE** 1998 Sexual differentiation and environmental endocrine disrupters. Baillieres Clin Endocrinol Metab 12:143-156

110. **Stillman RJ** 1982 In utero exposure to diethylstilbestrol: adverse effects on the reproductive tract and reproductive performance and male and female offspring. Am J Obstet Gynecol 142:905-921

111. **Norgil Damgaard I, Maria Main K, Toppari J, Skakkebaek NE** 2002 Impact of exposure to endocrine disrupters inutero and in childhood on adult reproduction. Best Practice & Research Clinical Endocrinology & Metabolism 16:289-309

112. **Wilcox AJ, Baird DD, Weinberg CR, Hornsby PP, Herbst AL** 1995 Fertility in Men Exposed Prenatally to Diethylstilbestrol. N Engl J Med 332:1411-1416

113. **Raman-Wilms L, Tseng AL, Wighardt S, Einarson TR, Koren G** 1995 Fetal genital effects of first-trimester sex hormone exposure: a meta-analysis. Obstet Gynecol 85:141-149

114. **Akingbemi BT, Hardy MP** 2001 Oestrogenic and antiandrogenic chemicals in the environment: effects on male reproductive health. Ann Med 33:391-403

115. **vom Saal FS, Cooke PS, Buchanan DL, Palanza P, Thayer KA, Nagel SC, Parmigiani S, Welshons WV** 1998 A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior. Toxicol Ind Health 14:239-260

116. **Cheek AO, McLachlan JA** 1998 Environmental hormones and the male reproductive system. J Androl 19:5-10

117. **Wilson JD** 1999 The Role of Androgens in Male Gender Role Behavior. Endocr Rev 20:726-737

118. **Pflieger-Bruss S, Schuppe HC, Schill WB** 2004 The male reproductive system and its susceptibility to endocrine disrupting chemicals. Andrologia 36:337-345

119. **Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ Jr, Jegou B, Jensen TK, Jouannet P, Keiding N, Leffers H, McLachlan JA, Meyer O, Muller J, Rajpert-De Meyts E, Scheike T, Sharpe R, Sumpter J, Skakkebaek NE** 1996 Male reproductive health and environmental xenoestrogens. Environ Health Perspect 104:741-803

120. **Stratakis CA, Vottero A, Brodie A, Kirschner LS, DeAtkine D, Lu Q, Yue W, Mitsiades CS, Flor AW, Chrousos GP** 1998 The Aromatase Excess Syndrome Is Associated with Feminization of Both Sexes and Autosomal Dominant Transmission of Aberrant P450 Aromatase Gene Transcription. Journal of Clinical Endocrinology Metabolism 83:1348-1357

121. **Borer JG, Tan PE, Diamond DA** 2000 The spectrum of Sertoli cell tumors in children. Urol Clin North Am 27:529-541

122. **Harms D, Kock LR** 1997 Testicular juvenile granulosa cell and Sertoli cell tumours: a clinicopathological study of 29 cases from the Kiel Paediatric Tumour Registry. Virchows Archiv V430:301-309

123. **Rushton HG, Belman AB** 1993 Testis-sparing surgery for benign lesions of the prepubertal testis. Urol Clin North Am 20:27-37

124. **Kaplan GW, Cromie WJ, Kelalis PP, Silber I, Tank ES Jr** 1986 Gonadal stromal tumors: a report of the Prepubertal Testicular Tumor Registry. J Urol 136:300-302

125. **Holm M, Rajpert-De Meyts E, Andersson AM, Skakkebaek NE** 2003 Leydig cell micronodules are a common finding in testicular biopsies from men with impaired spermatogenesis and are associated with decreased testosterone/LH ratio. J Pathol 199:378-386

126. **Shenker A, Laue L, Kosugi S, Merendino JJ, Minegishi T, Cutler GB** 1993 A constitutively activating mutation of the luteinizing hormone receptor in familial male precocious puberty. Nature 365:652-654

127. **Liu G, Duranteau L, Carel JC, Monroe J, Doyle DA, Shenker A** 1999 Leydig-Cell Tumors Caused by an Activating Mutation of the Gene Encoding the Luteinizing Hormone Receptor. N Engl J Med 341:1731-1736

128. **Iiri T, Herzmark P, Nakamoto JM, Van Dop C, Bourne HR** 1994 Rapid GDP release from Gs[alpha] in patients with gain and loss of endocrine function. Nature 371:164-168

129. **Villares Fragoso MC, Latronico AC, Carvalho FM, Zerbini MC, Marcondes JAM, Araujo LMB, Lando VS, Frazzatto ET, Mendonca BB, Villares SM** 1998 Activating Mutation of the Stimulatory G Protein (gsp) as a Putative Cause of Ovarian and Testicular Human Stromal Leydig Cell Tumors. Journal of Clinical Endocrinology Metabolism 83:2074-2078

130. **Themmen AP, Brunner HG** 1996 Luteinizing hormone receptor mutations and sex differentiation. Eur J Endocrinol 134:533-540

131. **Dilworth JP, Farrow GM, Oesterling JE** 1991 Non-germ cell tumors of testis. Urology 37:399-417

132. **Rich MA, Keating MA** 2000 Leydig cell tumors and tumors associated with congenital adrenal hyperplasia. Urol Clin North Am 27:519-528

133. **Kim I, Young RH, Scully RE** 1985 Leydig cell tumors of the testis. A clinicopathological analysis of 40 cases and review of the literature. Am J Surg Pathol 9:177-192

134. **Bertram KA, Bratloff B, Hodges GF, Davidson H** 1991 Treatment of malignant Leydig cell tumor. Cancer 68:2324-2329

135. **Cheville JC, Sebo TJ, Lager DJ, Bostwick DG, Farrow GM** 1998 Leydig cell tumor of the testis: a clinicopathologic, DNA content, and MIB-1 comparison of nonmetastasizing and metastasizing tumors. Am J Surg Pathol 22:1361-1367

136. **Iwata H, Hirouchi Y, Koike Y** 1991 Historical control data of non-neoplastic and neoplastic lesions in F-344/DuCrj. J Appl Toxicol 4:24

137. **Boorman G, Eustis S, Montgomery C, Elwell M, Mackenzie W** 1990 Pathology of the Fisher rat. Academic Press, San Diego405-418

138. **Jones TC, Mohr U, Hunt RD** 1987 Monographs on phatology of laboratory animals Genital System. Spring -Verlag, Berlin184-192

139. **Fowler KA, Gill K, Kirma N, Dillehay DL, Tekmal RR** 2000 Overexpression of Aromatase Leads to Development of Testicular Leydig Cell Tumors : An in Vivo Model for Hormone-Mediated TesticularCancer. Am J Pathol 156:347-353

140. **Carpino A, Rago V, Pezzi V, Carani C, Ando S** 2007 Detection of aromatase and estrogen receptors (ER{alpha}, ER{beta}1, ER{beta}2) in human Leydig cell tumor. Eur J Endocrinol 157:239-244

141. **Düe W, Dieckmann KP, Loy V, Stein H** 1989 Immunohistological determination of oestrogen receptor, progesterone receptor, and intermediate filaments in Leydig cell tumours, Leydig cell hyperplasia, and normal Leydig cells of the human testis. J Pathol 157:225-234

142. **Turner KJ, Macpherson S, Millar MR, McNeilly AS, Williams K, Cranfield M, Groome NP, Sharpe RM, Fraser HM, Saunders PT** 2002 Development and validation of a new monoclonal antibody to mammalian aromatase. J Endocrinol 172:21-30

143. **Ho GT, Gardner H, Mostofi K, DeWolf WC, Loughlin KR, Morgentaler A** 1994 The effect of testicular nongerm cell tumors on local spermatogenesis. Fertil Steril 62:162-166

144. **Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A.** 1993 Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell 75:59-72

145. **Pollak MN, Schernhammer ES, Hankinson SE** 2004 Insulin-like growth factors and neoplasia. Nat Rev Cancer 4:505-518

146. **Khandwala HM, McCutcheon IE, Flyvbjerg A, Friend KE** 2000 The Effects of Insulin-Like Growth Factors on Tumorigenesis and Neoplastic Growth. Endocr Rev 21:215-244

147. **Moschos SJ, Mantzoros CS** 2002 The role of the IGF system in cancer: from basic to clinical studies and clinical applications. Oncology 63:317-332

148. **Rinderknecht E, Humbel RE** 1978 The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. J Biol Chem 253:2769-2776

149. **Rinderknecht E, Humbel RE** 1978 Primary structure of human insulin-like growth factor II. FEBS Letters 89:283-286

150. **Isaacs N, James R, Niall H, Bryant-Greenwood G, Dodson G, Evans A, North AC** 1978 Relaxin and its structural relationship to insulin. Nature 271:278-281

151. **Kawakami A, Kataoka H, Oka T, Mizoguchi A, Kimura-Kawakami M, Adachi T, Iwami M, Nagasawa H, Suzuki A, Ishizaki H** 1990 Molecular cloning of the Bombyx mori prothoracicotropic hormone. Science 247:1333-1335

152. **Smit AB, Vreugdenhil E, Ebberink RHM, Geraerts WPM, Klootwijk J, Joosse J** 1988 Growth-controlling molluscan neurons produce the precursor of an insulin-related peptide. Nature 331:535-538

153. **Lagueux M, Lagueux M, Lwoff L, Meister M, Goltzené F, Hoffmann JA** 1990 cDNAs from neurosecretory cells of brains of Locusta migratoria (Insecta, Orthoptera) encoding a novel member of the superfamily of insulins. Eur J Biochem 187:254

154. **Shimatsu A, Rotwein P** 1987 Sequence of two rat insulin-like growth factor I mRNAs differing within the 5' untranslated region. Nucl Acids Res 15:7196

155. **Zapf J, Froesch ER** 1986 Insulin-like growth factors/somatomedins: structure, secretion, biological actions and physiological role. Horm Res 24:121-130

156. **Koval A, Kulik V, Duguay S, Plisetskaya E, Adamo ML, Roberts CT Jr, Leroith D, Kavsan V** 1994 Characterization of a salmon insulin-like growth factor I promoter. DNA Cell Biol 13:1057-1062

157. **Chan SJ, Cao Q, Steiner DF** 1990 Evolution of the Insulin Superfamily: Cloning of a Hybrid Insulin/Insulin- Like Growth Factor cDNA from Amphioxus. PNAS 87:9319-9323

158. **Gronowski AM, Rotwein P** 1995 Rapid changes in gene expression after in vivo growth hormone treatment. Endocrinology 136:4741-4748

159. **Rabkin R.** 1997 Nutrient regulation of insulin-like growth factor-I. Miner Electrolyte Metab 23:157-160

160. **D'Ercole AJ** 1987 Somatomedins/insulin-like growth factors and fetal growth. J Dev Physiol 9:481-495

161. **Sepp-Lorenzino L** 1998 Structure and function of the insulin-like growth factor I receptor. Breast Cancer Research and Treatment 47:235-253

162. **Sundaresan S, Francke U** 1989 Insulin-like growth factor I receptor gene is concordant with c-Fes protooncogene and mouse chromosome 7 in somatic cell hybrids. Somat Cell Mol Genet 15:373-376

163. **Werner H, Woloschak M, Adamo M, Shen-Orr Z, Roberts CT, LeRoith D** 1989 Developmental Regulation of the Rat Insulin-Like Growth Factor I Receptor Gene. PNAS 86:7451-7455

164. **Santos A, Yusta B, Fernández-Moreno MD, Blázquez E** 1994 Expression of insulin-like growth factor-I (IGF-I) receptor gene in rat brain and liver during development and in regenerating adult rat liver. Mol Cell Endocrinol 101:85-93

165. **Werner H, Karnieli E, Rauscher FJ, III, LeRoith D** 1996 Wild-type and mutant p53 differentially regulate transcription of the insulin-like growth factor I receptorágene. PNAS 93:8318-8323

166. **Burant CF, Treutelaar MK, Allen KD, Sens DA, Buse MG** 1987 Comparison of insulin and insulin-like growth factor I receptors from rat skeletal muscle and L-6 myocytes. Biochemical and Biophysical Research Communications 147:100-107

167. **Siddle K, Soos MA, Field CE, Navé BT** 1994 Hybrid and atypical insulin/insulin-like growth factor I receptors. Horm Res 41:56-64

168. **Pandini G, Frasca F, Mineo R, Sciacca L, Vigneri R, Belfiore A** 2002 Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved. J Biol Chem 277:39684-39695

169. **Braulke T** 1999 Type-2 IGF receptor: a multi-ligand binding protein. Horm Metab Res 31:242-246

170. **Blanchard F, Duplomb L, Raher S, Vusio P, Hoflack B, Jacques Y, Godard A** 1999 Mannose 6-Phosphate/Insulin-like growth factor II receptor mediates internalization and degradation of leukemia inhibitory factor but not signal transduction. J Biol Chem 274:24685-24693

171. **Morgan DO, Edman JC, Standring DN, Fried VA, Smith MC, Roth RA, Rutter WJ** 1987 Insulin-like growth factor II receptor as a multifunctional binding protein. Nature 329:301-307

172. **von Figura K, Steckel F, Conary J, Hasilik A, Shaw E** 1986 Heterogeneity in late-onset metachromatic leukodystrophy. Effect of inhibitors of cysteine proteinases. Am J Hum Genet 39:371-382

173. **Denley A, Bonython ER, Booker GW, Cosgrove LJ, Forbes BE, Ward CW, Wallace JC** 2004 Structural determinants for high-affinity binding of insulin-like growth factor II to insulin receptor (IR)-A, the exon 11 minus isoform of the IR. Mol Endocrinol 18:2502-2512

174. **Frasca F, Pandini G, Scalia P, Sciacca L, Mineo R, Costantino A, Goldfine ID, Belfiore A** 1999 Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. Mol Cell Biol 19:3278-3288

175. **Jones JI, Clemmons DR** 1995 Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev 16:3-34

176. **Firth SM, Baxter RC** 2002 Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev 23:824-854

177. **Lalou C, Sawamura S, Segovia B, Ogawa Y, Binoux M.** 1997 Proteolytic fragments of insulin-like growth factor binding protein-3: N-terminal sequences and relationships between structure and biological activity. C R Acad Sci III 320:621-628

178. **Schütt BS, Langkamp M, Rauschnabel U, Ranke MB, Elmlinger MW** 2004 Integrin-mediated action of insulin-like growth factor binding protein-2 in tumor cells. J Mol Endocrinol 32:859-868

179. **Schedlich LJ, Young TF, Firth SM, Baxter RC** 1998 Insulin-like growth factor-binding protein (IGFBP)-3 and IGFBP-5 share a common nuclear transport pathway in T47D human breast carcinoma cells. J Biol Chem 273:18347-18352

180. **Wraight CJ, Liepe IJ, White PJ, Hibbs AR, Werther GA** 1998 Intranuclear localization of insulin-like growth factor binding protein-3 (IGFBP-3) during cell division in human keratinocytes. J Invest Dermatol 111:239-242

181. **Hoeflich A, Reisinger R, Schuett BS, Elmlinger MW, Russo VC, Vargas GA, Jehle PM, Lahm H, Renner-Müller I, Wolf E** 2004 Peri/nuclear localization of intact insulin-like growth factor binding protein-2 and a distinct carboxyl-terminal IGFBP-2 fragment in vivo. Biochem Biophys Res Commun 324:705-710

182. **Lejeune H, Chuzel F, Thomas T, Avallet O, Habert R, Durand P, Saez J.** 1996 Paracrine regulation of Leydig cells. Ann Endocrinol (Paris) 57:55-63

183. **Grizard G, Fournet M, Rigaudière N, Lombard-Vignon N, Grizard J** 1991 Insulin binding to Leydig cells and insulin levels in testicular interstitial fluid at different stages of development in the rat. J Endocrinol 128:375-381

184. **Saez JM** 1994 Leydig cells: endocrine, paracrine, and autocrine regulation. Endocr Rev 15:574-626

185. **Handelsman DJ, Spaliviero JA, Scott CD, Baxter RC** 1985 Identification of insulin-like growth factor-I and its receptors in the rat testis. Acta Endocrinol 109:543-549

186. **Hansson HA, Billig H, Isgaard J.** 1989 Insulin-like growth factor I in the developing and mature rat testis: immunohistochemical aspects. Biol Reprod 40:1321-1328

187. **Lin T, Wang DL, Calkins JH, Guo H, Chi R, Housley PR** 1990 Regulation of insulin-like growth factor-I messenger ribonucleic acid expression in Leydig cells. Mol Cell Endocrinol 73:147-152

188. **Vannelli BG, Barni T, Orlando C, Natali A, Serio M, Balboni GC** 1988 Insulin-like growth factor-I (IGF-I) and IGF-I receptor in human testis: an immunohistochemical study. Fertil Steril 49:666-669

189. **Cailleau J, Vermeire S, Verhoeven G** 1990 Independent control of the production of insulin-like growth factor I and its binding protein by cultured testicular cells. Mol Cell Endocrinol 69:79-89

190. **Naville D, Chatelain PG, Avallet O, Saez JM** 1990 Control of production of insulin-like growth factor I by pig Leydig and Sertoli cells cultured alone or together. Cell-cell interactions. Mol Cell Endocrinol 70:217-224

191. **Lin T, Haskell J, Vinson N, Terracio L.** 1986 Characterization of insulin and insulin-like growth factor I receptors of purified Leydig cells and their role in steroidogenesis in primary culture: a comparative study. Endocrinology 119:1641-1647

192. **Perrard-Sapori MH, Chatelain PG, Jaillard C, Saez JM** 1987 Characterization and regulation of somatomedin-C/insulin-like growth factor I (Sm-C/IGF-I) receptors on cultured pig Leydig cells. Effects of Sm-C/IGF-I on luteotropin receptors and steroidogenesis. Eur J Biochem 165:209-214

193. **Lin T** 1995 Regulation of Leydig cell function by insulin-like growth factor-I and binding proteins. Journal of Andrology 16:193-196

194. **Kasson BG, Hsueh AJW** 1987 Insulin-like growth factor-I augments gonadotropin-stimulated androgen biosynthesis by cultured rat testicular cells. Molecular and Cellular Endocrinology 52:27-34

195. **Bernier M, Chatelain P, Mather JP, Saez JM** 1986 Regulation of gonadotropin receptors, gonadotropin responsiveness, and cell multiplication by somatomedin-C and insulin in cultured pig Leydig cells. J Cell Physiol 129:257-263

196. **Chuzel F, Clark AM, Avallet O, Saez JM** 1996 Transcriptional regulation of the lutropin/human choriogonadotropin receptor and three enzymes of steroidogenesis by growth factors in cultured pig Leydig cells. Eur J Biochem 239:8-16

197. **Rigaudiere N, Grizard G, Boucher D** 1989 Aromatase activity in purified Leydig cells from adult rat. Comparative effects of insulin, IGF-I, and hCG. Acta Endocrinol (Copenh) 121:677-685

198. **Saez JM, Chatelain PG, Perrard-Sapori MH, Jaillard C, Naville D** 1988 Differentiating effects of somatomedin-C/insulin-like growth factor I and insulin on Leydig and Sertoli cell functions. Reprod Nutr Dev 28:989-1008

199. **Lin T, Vinson N, Haskett J, Murono EP** 1987 Induction of 3 beta-hydroxysteroid dehydrogenase activity by insulin-like growth factor-I in primary culture of purified Leydig cells. Adv Exp Med Biol 219:603-607

200. **Spiteri-Grech J, Nieschlag E.** 1992 The role of growth hormone and insulin-like growth factor I in the regulation of male reproductive function. Horm Res 38:22-27

201. **Baker J, Hardy MP, Zhou J, Bondy C, Lupu F, Bellve AR, Efstratiadis A.** 1996 Effects of an Igf1 gene null mutation on mouse reproduction. Mol Endocrinol 10:903-918

202. **Wang GM, O'Shaughnessy PJ, Chubb C, Robaire B, Hardy MP** 2003 Effects of Insulin-Like Growth Factor I on Steroidogenic Enzyme Expression Levels in Mouse Leydig Cells. Endocrinology 144:5058-5064

203. **Lin D, Sugawara T, Strauss JF, III, Clark BJ, Stocco DM, Saenger P, Rogol A, Miller WL** 1995 Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. Science 267:1828-1831

204. **Clark BJ, Combs R, Hales KH, Hales DB, Stocco DM** 1997 Inhibition of transcription affects synthesis of steroidogenic acute regulatory protein and steroidogenesis in MA-10 mouse Leydig tumor cells. Endocrinology 138:4893-4901

205. **Baxter RC** 2000 Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. Am J Physiol Endocrinol Metab 278:967-976

206. **Hankinson SE, Schernhammer ES** 2003 Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. Breast Dis 17:27-40

207. **Kaaks R, Lundin E, Rinaldi S, Manjer J, Biessy C, Söderberg S, Lenner P, Janzon L, Riboli E, Berglund G, Hallmans G** 2002 Prospective study of IGF-I,

IGF-binding proteins, and breast cancer risk, in northern and southern Sweden. Cancer Causes Control 13:307-316

208. **Pollak MN, Perdue JF, Margolese RG, Baer K, Richard M** 1987 Presence of somatomedin receptors on primary human breast and colon carcinomas. Cancer Lett 38:223-230

209. **Lambert S, Collette J, Gillis J, Franchimont P, Desaive C, Gol-Winkler R** 1991 Tumor IGF-II content in a patient with a colon adenocarcinoma correlates with abnormal expression of the gene. Int J Cancer 48:826-830

210. **Lahm H, Suardet L, Laurent PL, Fischer JR, Ceyhan A, Givel JC, Odartchenko N** 1992 Growth regulation and co-stimulation of human colorectal cancer cell lines by insulin-like growth factor I, II and transforming growth factor alpha. Br J Cancer 65:-341

211. **Lahm H, Amstad P, Wyniger J, Yilmaz A, Fischer JR, Schreyer M, Givel JC** 1994 Blockade of the insulin-like growth-factor-I receptor inhibits growth of human colorectal cancer cells: evidence of a functional IGF-II-mediated autocrine loop. Int J Cancer 58:452-459

212. **Brunner JE, Johnson CC, Zafar S, Peterson EL, Brunner JF, Mellinger RC** 1990 Colon cancer and polyps in acromegaly: increased risk associated with family history of colon cancer. Clin Endocrinol (Oxf) 32:65-71

213. **Michell NP, Dent S, Langman MJ, Eggo MC** 1997 Insulin-like growth factor binding proteins as mediators of IGF-I effects on colon cancer cell proliferation. Growth Factors 14:269-277

214. **Rajah R, Valentinis B, Cohen P** 1997 Insulin-like growth factor (IGF)-binding protein-3 induces apoptosis and mediates the effects of transforming growth factor-beta1 on programmed cell death through a p53- and IGF-independent mechanism. J Biol Chem 272:12181-12188

215. **Kelley KM, Oh Y, Gargosky SE, Gucev Z, Matsumoto T, Hwa V, Ng L, Simpson DM, Rosenfeld RG** 1996 Insulin-like growth factor-binding proteins (IGFBPs) and their regulatory dynamics. Int J Biochem Cell Biol 28:619-637

216. **Pollak M, Costantino J, Polychronakos C, Blauer SA, Guyda H, Redmond C, Fisher B, Margolese R** 1990 Effect of tamoxifen on serum insulin like growth factor I levels in stage I breast cancer patients. J Natl Cancer Inst 82:1693-1697

217. **Hla T, Neilson K** 1992 Human Cyclooxygenase-2 cDNA. PNAS 89:7384-7388

218. **Smith CJ, Zhang Y, Koboldt CM, Muhammad J, Zweifel BS, Shaffer A, Talley JJ, Masferrer JL, Seibert K, Isakson PC** 1998 Pharmacological analysis of cyclooxygenase-1 in inflammation. PNAS 95:13313-13318

219. **Chan CC, Boyce S, Brideau C, Charleson S, Cromlish W, Ethier D, Evans J, Ford-Hutchinson AW, Forrest MJ, Gauthier JY, Gordon R, Gresser M, Guay J, Kargman S, Kennedy B, Leblanc Y, Leger S, Mancini J, O'Neill GP, Ouellet M, Patrick D, Percival MD, Perrier H, Prasit P, Rodger I, Tagari P, Therien M, Vickers P, Visco D, Wang Z, Webb J, Wong E, Xu LJ, Young RN, Zamboni R, Riendeau D** 1999 Rofecoxib [Vioxx, MK-0966; 4-(4'-Methylsulfonylphenyl)-3-phenyl-2-(5H)-furanone]: A Potent and Orally Active Cyclooxygenase-2 Inhibitor. Pharmacological and Biochemical Profiles. J Pharmacol Exp Ther 290:551-560

220. **Riendeau D, Percival MD, Brideau C, Charleson S, Dube D, Ethier D, Falgueyret JP, Friesen RW, Gordon R, Greig G, Guay J, Mancini J, Ouellet M, Wong E, Xu L, Boyce S, Visco D, Girard Y, Prasit P, Zamboni R, Rodger IW, Gresser M, Ford-Hutchinson AW, Young RN, Chan CC** 2001 Etoricoxib (MK-0663): Preclinical Profile and Comparison with Other Agents That Selectively Inhibit Cyclooxygenase-2. J Pharmacol Exp Ther 296:558-566

221. **Lee SH, Soyoola E, Chanmugam P, Hart S, Sun W, Zhong H, Liou S, Simmons D, Hwang D** 1992 Selective expression of mitogen-inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide. J Biol Chem 267:25934-25938

222. **Eliopoulos AG, Dumitru CD, Wang CC, Cho J, Tsichlis PN** 2002 Induction of COX-2 by LPS in macrophages is regulated by Tpl2-dependent CREB activation signals. EMBO J 21:4831-4840

223. **Chen JJ, Huang WC, Chen CC** 2005 Transcriptional Regulation of Cyclooxygenase-2 in Response to Proteasome Inhibitors Involves Reactive Oxygen Species-mediated Signaling Pathway and Recruitment of CCAAT/Enhancer-binding Protein {delta} and CREB-binding Protein. Mol Biol Cell 16:5579-5591

224. **Deng WG, Zhu Y, Wu KK** 2004 Role of p300 and PCAF in regulating cyclooxygenase-2 promoter activation by inflammatory mediators. Blood 103:2135-2142

225. **Landino L, Crews B, Timmons M, Morrow J, Marnett L** 1996 Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandinábiosynthesis. PNAS 93:15069-15074

226. **Chin K, Kurashima Y, Ogura T, Tajiri H, Yoshida S, Esumi H** 1997 Induction of vascular endothelial growth factor by nitric oxide in human glioblastoma and hepatocellular carcinoma cells. Oncogene 15:437-442

227. **Salvemini D, Misko TP, Masferrer JL, Seibert K, Currie MG, Needleman P** 1993 Nitric Oxide Activates Cyclooxygenase Enzymes. PNAS 90:7240-7244

228. **Liu Y, Borchert GL, Phang JM** 2004 Polyoma Enhancer Activator 3, an Ets Transcription Factor, Mediates the Induction of Cyclooxygenase-2 by Nitric Oxide in Colorectal Cancer Cells. J Biol Chem 279:18694-18700

229. **Caivano M, Cohen P** 2000 Role of Mitogen-Activated Protein Kinase Cascades in Mediating Lipopolysaccharide-Stimulated Induction of Cyclooxygenase-2 and IL-1{beta} in RAW264 Macrophages. J Immunol 164:3018-3025

230. **Chun KS, Surh YJ** 2004 Signal transduction pathways regulating cyclooxygenase-2 expression: potential molecular targets for chemoprevention. Biochem Pharmacol 68:1089-1100

231. **Bachelor MA, Cooper SJ, Sikorski ET, Bowden GT** 2005 Inhibition of p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase decreases UVB-induced activator protein-1 and cyclooxygenase-2 in a SKH-1 hairless mouse model. Mol Cancer Res 3:90-99

232. **McEvoy AN, Bresnihan B, FitzGerald O, Murphy EP** 2004 Cyclooxygenase 2-derived prostaglandin E2 production by corticotropin-releasing hormone contributes to the activated cAMP response element binding protein content in rheumatoid arthritis synovial tissue. Arthritis Rheum 50:1132-1145

233. **Ramer R, Weinzierl U, Schwind B, Brune K, Hinz B** 2003 Ceramide is involved in r(+)-methanandamide-induced cyclooxygenase-2 expression in human neuroglioma cells. Mol Pharmacol 64:1189-1198

234. **Wu D, Marko M, Claycombe K, Paulson KE, Meydani SN** 2003 Ceramide-induced and age-associated increase in macrophage COX-2 expression is mediated through up-regulation of NF-kappa B activity. J Biol Chem 278:10983-10992

235. **Wang Q, Zhou Y, Wang X, Evers BM** 2006 Glycogen synthase kinase-3 is a negative regulator of extracellular signal-regulated kinase. Oncogene 25:43-50

236. **Katsanou V, Papadaki O, Milatos S, Blackshear PJ, Anderson P, Kollias G, Kontoyiannis DL** 2005 HuR as a negative posttranscriptional modulator in inflammation. Mol Cell 19:777-789

237. **Kim SF, Huri DA, Snyder SH** 2005 Inducible nitric oxide synthase binds, S-nitrosylates, and activates cyclooxygenase-2. Science 310:1966-1970

238. **Tuomisto TT, Riekkinen MS, Viita H, Levonen AL, Ylä-Herttuala S** 2005 Analysis of gene and protein expression during monocyte-macrophage differentiation and cholesterol loading--cDNA and protein array study. Atherosclerosis 180:283-291

239. **Burleigh ME, Babaev VR, Yancey PG, McCaleb JL, Oates JA, Morrow JD, Fazio S, Linton MF** 2005 Cyclooxygenase-2 promotes early atherosclerotic lesion formation in ApoE-deficient and C57BL/6 mice. J Mol Cell Cardiol 39:443-452

240. **Michelsen KS, Wong MH, Shah PK, Zhang W, Yano J, Doherty TM, Akira S, Rajavashisth TB, Arditi M** 2004 Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E. PNAS 101:10679-10684

241. **Shankavaram UT, Lai WC, Netzel-Arnett S, Mangan PR, Ardans JA, Caterina N, Stetler-Stevenson WG, Birkedal-Hansen H, Wahl LM** 2001 Monocyte membrane type 1-matrix metalloproteinase. Prostaglandin-dependent regulation and role in metalloproteinase-2 activation. J Biol Chem 276:19027-19032

242. **Launer L** 2003 Nonsteroidal anti-inflammatory drug use and the risk for Alzheimer's disease: dissecting the epidemiological evidence. Drugs 63:731-739

243. **Reddy BS, Rao CV, Seibert K** 1996 Evaluation of cyclooxygenase-2 inhibitor for potential chemopreventive properties in colon carcinogenesis. Cancer Res 56:4566-4569

244. **Rioux N, Castonguay A.** 1998 Prevention of NNK-induced lung tumorigenesis in A/J mice by acetylsalicylic acid and NS-398. Cancer Res 58:5354-5360

245. **Hara A, Yoshimi N, Niwa M, Ino N, Mori H** 1997 Apoptosis induced by NS-398, a selective cyclooxygenase-2 inhibitor, in human colorectal cancer cell lines. Jpn J Cancer Res 88:600-604

246. **Liu XH, Yao S, Kirschenbaum A, Levine AC** 1998 NS398, a selective cyclooxygenase-2 inhibitor, induces apoptosis and down-regulates bcl-2 expression in LNCaP cells. Cancer Res 58:4245-4249

247. **Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN** 1994 Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 107:1183-1188

248. **Ristimäki A, Honkanen N, Jänkälä H, Sipponen P, Härkönen M.** 1997 Expression of cyclooxygenase-2 in human gastric carcinoma. Cancer Res 57:1276-1280

249. **Zimmermann KC, Sarbia M, Weber AA, Borchard F, Gabbert HE, Schrör K.** 1999 Cyclooxygenase-2 expression in human esophageal carcinoma. Cancer Res 59:198-204

250. **Hida T, Yatabe Y, Achiwa H, Muramatsu H, Kozaki K, Nakamura S, Ogawa M, Mitsudomi T, Sugiura T, Takahashi T** 1998 Increased expression of cyclooxygenase 2 occurs frequently in human lung cancers, specifically in adenocarcinomas. Cancer Res 58:3761-3764

251. **Koga H, Sakisaka S, Ohishi M, Kawaguchi T, Taniguchi E, Sasatomi K, Harada M, Kusaba T, Tanaka M, Kimura R, Nakashima Y, Nakashima O, Kojiro M, Kurohiji T, Sata M** 1999 Expression of cyclooxygenase-2 in human hepatocellular carcinoma: relevance to tumor dedifferentiation. Hepatology 29:688-696

252. **Tucker ON, Dannenberg AJ, Yang EK, Zhang F, Teng L, Daly JM, Soslow RA, Masferrer JL, Woerner BM, Koki AT, Fahey TJ 3rd** 1999 Cyclooxygenase-2 expression is up-regulated in human pancreatic cancer. Cancer Res 59:987-990

253. **Frungieri MB, Weidinger S, Meineke V, Köhn FM, Mayerhofer A** 2002 Proliferative action of mast-cell tryptase is mediated by PAR2, COX2, prostaglandins, and PPARgamma : Possible relevance to human fibrotic disorders. Proc Natl Acad Sci U S A 99:15072-15077

254. **Hase T, Yoshimura R, Matsuyama M, Kawahito Y, Wada S, Tsuchida K, Sano H, Nakatani T** 2003 Cyclooxygenase-1 and -2 in human testicular tumours. Eur J Cancer 39:2043-2049

255. **Jiang WG, Douglas-Jones A, Mansel RE** 2003 Levels of expression of lipoxygenases and cyclooxygenase-2 in human breast cancer. Prostaglandins Leukot Essent Fatty Acids 69:275-281

256. **Ristimäki A, Sivula A, Lundin J, Lundin M, Salminen T, Haglund C, Joensuu H, Isola J.** 2002 Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. Cancer Res 62:632-635

257. **Kirkpatrick K, Ogunkolade W, Elkak A, Bustin S, Jenkins P, Ghilchik M, Mokbel K** 2002 The mRNA expression of cyclo-oxygenase-2 (COX-2) and

vascular endothelial growth factor (VEGF) in human breast cancer. Curr Med Res Opin 18:237-241

258. **Ranger GS, Thomas V, Jewell A, Mokbel K** 2004 Elevated cyclooxygenase-2 expression correlates with distant metastases in breast cancer. Anticancer Res 24:2349-2351

259. **Brueggemeier RW, Quinn AL, Parrett ML, Joarder FS, Harris RE, Robertson FM** 1999 Correlation of aromatase and cyclooxygenase gene expression in human breast cancer specimens. Cancer Lett 140:27-35

260. **Díaz-Cruz ES, Shapiro CL, Brueggemeier RW** 2005 Cyclooxygenase inhibitors suppress aromatase expression and activity in breast cancer cells. J Clin Endocrinol Metab 90:2563-2570

261. **Prosperi JR, Robertson FM.** 2006 Cyclooxygenase-2 directly regulates gene expression of P450 Cyp19 aromatase promoter regions pII, pI.3 and pI.7 and estradiol production in human breast tumor cells. Prostaglandins Other Lipid Mediat 81:55-70

262. **Nolan RD, Danilowicz RM, Eling TE.** 1988 Role of arachidonic acid metabolism in the mitogenic response of BALB/c 3T3 fibroblasts to epidermal growth factor. Mol Pharmacol 33:650-656

263. **Bandyopadhyay GK, Imagawa W, Wallace D, Nandi S** 1987 Linoleate metabolites enhance the in vitro proliferative response of mouse mammary epithelial cells to epidermal growth factor. J Biol Chem 262:2750-2756

264. **Stolina M, Sharma S, Lin Y, Dohadwala M, Gardner B, Luo J, Zhu L, Kronenberg M, Miller PW, Portanova J, Lee JC, Dubinett SM.** 2000 Specific inhibition of cyclooxygenase 2 restores antitumor reactivity by altering the balance of IL-10 and IL-12 synthesis. J Immunol 164:361-370

265. **Rolland PH, Martin PM, Jacquemier J, Rolland AM, Toga M.** 1980 Prostaglandin in human breast cancer: Evidence suggesting that an elevated prostaglandin production is a marker of high metastatic potential for neoplastic cells. J Natl Cancer Inst 64:1061-1070

266. **Schrey MP, Patel KV** 1995 Prostaglandin E2 production and metabolism in human breast cancer cells and breast fibroblasts. Regulation by inflammatory mediators. Br J Cancer 72:1412-1419

267. **Zhao Y, Agarwal VR, Mendelson CR, Simpson ER** 1996 Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE2 via cyclic AMP, leading to

activation of promoter II of the CYP19 (aromatase) gene. Endocrinology 137:5739-5742

268. **Purohit A, Newman SP, Reed MJ** 2002 The role of cytokines in regulating estrogen synthesis: implications for the etiology of breast cancer. Breast Cancer Res 4:65-69

269. **Purohit A, Reed MJ** 2002 Regulation of estrogen synthesis in postmenopausal women. Steroids 67:979-983

270. **Coleman RA, Smith WL, Narumiya S.** 1994 International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. Pharmacol Rev 46:205-229

271. **Funk CD, Furci L, FitzGerald GA, Grygorczyk R, Rochette C, Bayne MA, Abramovitz M, Adam M, Metters KM** 1993 Cloning and expression of a cDNA for the human prostaglandin E receptor EP1 subtype. J Biol Chem 268:26767-26772

272. **Regan JW, Bailey TJ, Pepperl DJ, Pierce KL, Bogardus AM, Donello JE, Fairbairn CE, Kedzie KM, Woodward DF, Gil DW** 1994 Cloning of a novel human prostaglandin receptor with characteristics of the pharmacologically defined EP2 subtype. Mol Pharmacol 46:213-220

273. **Kunapuli SP, Fen Mao G, Bastepe M, Liu-Chen LY, Li S, Cheung PP, DeRiel JK, Ashby B.** 1994 Cloning and expression of a prostaglandin E receptor EP3 subtype from human erythroleukaemia cells. Biochem J 298:263-267

274. **Breyer RM, Davis LS, Nian C, Redha R, Stillman B, Jacobson HR, Breyer MD.** 1996 Cloning and expression of the rabbit prostaglandin EP4 receptor. Am J Physiol 270:485-493

275. **Breyer RM, Bagdassarian CK, Myers SA, Breyer MD.** 2001 Prostanoid receptors: subtypes and signaling. Annu Rev Pharmacol Toxicol 41:661-690

276. **Richards JA, Brueggemeier RW.** 2003 Prostaglandin E2 regulates aromatase activity and expression in human adipose stromal cells via two distinct receptor subtypes. J Clin Endocrinol Metab 88:2010-2816

277. **Lephart ED, Simpson ER.** 1991 Assay of aromatase activity. Methods Enzymol 206:477-483

278. **Bradford MM** 1976 A rapid and sensitive method for thequantitation of microgram quantities of protein utilizing theprinciple of protein-dye binding. Analytical Biochemistry 72:248-254

279. **Wolanska M, Bankowski E** 2004 An accumulation of insulin-like growth factor I (IGF-I) in human myometrium and uterine leiomyomas in various stages of tumour growth. Eur Cytokine Netw 15:359-363

280. **Shi SR, Key ME, Kalra KL** 1991 Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem 39:741-748

281. **Coleman GL, Barthold W, Osbaldiston GW, Foster SJ, Jonas AM** 1977 Pathological changes during aging in barrier-reared Fischer 344 male rats. J Gerontol 32:258-278

282. **Jacobs BB, Huseby RA** 1967 Neoplasms occurring in aged Fischer rats, with special reference to testicular, uterine and thyroid tumors. J Natl Cancer Inst303-309

283. **Jo Y, Stocco DM** 2004 Regulation of Steroidogenesis and Steroidogenic Acute Regulatory Protein in R2C Cells by DAX-1 (Dosage-Sensitive Sex Reversal, Adrenal Hypoplasia Congenita, Critical Region on the X Chromosome, Gene-1). Endocrinology 145:5629-5637

284. **LaVoie HA, Garmey JC, Veldhuis JD** 1999 Mechanisms of Insulin-Like Growth Factor I Augmentation of Follicle-Stimulating Hormone-Induced Porcine Steroidogenic Acute Regulatory Protein Gene Promoter Activity in Granulosa Cells. Endocrinology 140:146-153

285. **Pomerantz D** 1980 Developmental Changes in the Ability of Follicle Stimulating Hormone to Stimulate Estrogen Synthesis in vivo by the Testis of the Rat. Biol Reprod 23:948-954

286. **Kmicikiewicz I, Krezolek A, Bilinska B** 1997 The effects of aromatase inhibitor on basal and testosterone-supplemented estradiol secretion by Leydig cells in vitro. Exp Clin Endocrinol Diabetes 105:113-118

287. **Payne AH, Kelch RP, Musich SS, Halpern ME** 1976 Intratesticular site of aromatization in the human. J Clin Endocrinol Metab 42:1081-1087

288. **Raeside JI, Renaud RL** 1983 Estrogen and androgen production by purified Leydig cells of mature boars. Biol Reprod 28:727-733

289. **Bilinska B, Lesniak M, Schmalz B** 1997 Are ovine Leydig cells able to aromatize androgens?. Reprod Fertil Dev 9:193-199

290. **Valensi P, Coussieu C, Pauwles A, Attali JR, Kemeny JL, Amouroux J, Sebaoun J** 1987 Feminizing Leydig cell tumor: endocrine and incubation studies. J Endocrinol Invest 10:187-193

291. **Saunders PT, Fisher JS, Sharpe RM, Millar MR** 1998 Expression of oestrogen receptor beta (ER beta) occurs in multiple cell types, including some germ cells, in the rat testis. J Endocrinol 156:R13-R17

292. **Prall OWJ, Sarcevic B, Musgrove EA, Watts CKW, Sutherland RL** 1997 Estrogen-induced Activation of Cdk4 and Cdk2 during G1-S Phase Progression Is Accompanied by Increased Cyclin D1 Expression and Decreased Cyclin-dependent Kinase Inhibitor Association with Cyclin E-Cdk2. J Biol Chem 272:10882-10894

293. **Hatano O, Takayama K, Imai T, Waterman MR, Takakusu A, Omura T, Morohashi K** 1994 Sex-dependent expression of a transcription factor, Ad4BP, regulating steroidogenic P-450 genes in the gonads during prenatal and postnatal rat development. Development 120:2787-2797

294. **Desclozeaux M, Krylova IN, Horn F, Fletterick RJ, Ingraham HA** 2002 Phosphorylation and Intramolecular Stabilization of the Ligand Binding Domain in the Nuclear Receptor Steroidogenic Factor 1. Mol Cell Biol 22:7193-7203

295. **Dufau ML, Winters CA, Hattori M, Aquilano D, Baranao JL, Nozu K, Baukal A, Catt KJ** 1984 Hormonal regulation of androgen production by the Leydig cell. J Steroid Biochem 20:161-173

296. **Jo Y, King SR, Khan SA, Stocco DM** 2005 Involvement of Protein Kinase C and Cyclic Adenosine 3',5'-Monophosphate-Dependent Kinase in Steroidogenic Acute Regulatory Protein Expression and Steroid Biosynthesis in Leydig Cells. Biol Reprod 73:244-255

297. **Rao RM, Jo Y, Leers-Sucheta S, Bose HS, Miller WL, Azhar S, Stocco DM** 2003 Differential Regulation of Steroid Hormone Biosynthesis in R2C and MA-10 Leydig Tumor Cells: Role of SR-B1-Mediated Selective Cholesteryl Ester Transport. Biol Reprod 68:114-121

298. **Nagpal ML, Wang D, Calkins JH, Chang WW, Lin T.** 1991 Human chorionic gonadotropin up-regulates insulin-like growth factor-I receptor gene expression of Leydig cells. Endocrinology 129:2820-2826

299. **Gelber SJ, Hardy MP, Mendis-Handagama SM, Casella SJ** 1992 Effects of insulin-like growth factor-I on androgen production by highly purified pubertal and adult rat Leydig cells. J Androl 13:125-130

300. **Lin T, Wang D, Hu J, Stocco DM** 1998 Upregulation of human chorionic gonadotrophin-induced steroidogenic acute regulatory protein by insulin-like growth factor-I in rat Leydig cells. Endocrine 8:73-78

301. **Kojima I, Mogami H, Shibata H, Ogata E** 1993 Role of calcium entry and protein kinase C in the progression activity of insulin-like growth factor-I in Balb/c 3T3 cells. J Biol Chem 268:10003-10006

302. **Song KH, Park YY, Park KC, Hong CY, Park JH, Shong M, Lee K, Choi HS** 2004 The Atypical Orphan Nuclear Receptor DAX-1 Interacts with Orphan Nuclear Receptor Nur77 and Represses Its Transactivation. Mol Endocrinol 18:1929-1940

303. **Brodie A, Lu Q, Nakamura J** 1997 Aromatase in the normal breast and breast cancer. J Steroid Biochem Mol Biol 61:281-286

304. **Miller WR, Mullen P, Sourdaine P, Watson C, Dixon JM, Telford J** 1997 Regulation of aromatase activity within the breast. J Steroid Biochem Mol Biol 61:192-202

305. **Harada N, Utsumi T, Takagi Y** 1993 Tissue-specific expression of the human aromatase cytochrome P-450 gene by alternative use of multiple exons 1 and promoters, and switching of tissue-specific exons 1 in carcinogenesis. Proc Natl Acad Sci U S A 90:11312-11316

306. **Zhou C, Zhou D, Esteban J, Murai J, Siiteri PK, Wilczynski S, Chen S.** 1996 Aromatase gene expression and its exon I usage in human breast tumors. Detection of aromatase messenger RNA by reverse transcription-polymerase chain reaction. J Steroid Biochem Mol Biol 59:163-171

307. **Irahara N, Miyoshi Y, Taguchi T, Tamaki Y, Noguchi S.** 2006 Quantitative analysis of aromatase mRNA expression derived from various promoters (I.4, I.3, PII and I.7) and its association with expression of TNF-alpha, IL-6 and COX-2 mRNAs in human breast cancer. Int J Cancer 118:1915-1921

308. **Reddy GP, Prasad M, Sailesh S, Kumar YV, Reddanna P** 1992 The production of arachidonic acid metabolites in rat testis. Prostaglandins 44:497-507

309. **Winnall WR, Ali U, O'Bryan MK, Hirst JJ, Whiley PA, Muir JA, Hedger MP** 2007 Constitutive expression of prostaglandin-endoperoxide synthase 2 by somatic

and spermatogenic cells is responsible for prostaglandin E2 production in the adult rat testis. Biol Reprod 76:759-768

310. **Lazarus M, Munday CJ, Eguchi N, Matsumoto S, Killian GJ, Kubata BK, Urade Y** 2002 Immunohistochemical localization of microsomal PGE synthase-1 and cyclooxygenases in male mouse reproductive organs. Endocrinology 143:2410-2419

311. **Gobbetti A, Zerani M** 2002 Pituitary adenylate cyclase-activating polypeptide induces testicular testosterone synthesis through PGE(2) mediation in crested newt, Triturus carnifex. J Exp Zool 293:73-80

312. **Shao J, Sheng H, Inoue H, Morrow JD, DuBois RN.** 2000 Regulation of constitutive cyclooxygenase-2 expression in colon carcinoma cells. J Biol Chem 275:33951-33956

313. **Fitzpatrick SL, Richards JS.** 1993 cis-acting elements of the rat aromatase promoter required for cyclic adenosine 3',5'-monophosphate induction in ovarian granulosa cells and constitutive expression in R2C Leydig cells. Mol Endocrinol 7:341-354

314. **Steinbach G, Lynch PM, Phillips RK, Wallace MH, Hawk E, Gordon GB, Wakabayashi N, Saunders B, Shen Y, Fujimura T, Su LK, Levin B.** 2000 The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. N Engl J Med 342:1946-1952

315. **Bertagnolli MM, Eagle CJ, Zauber AG, Redston M, Solomon SD, Kim K, Tang J, Rosenstein RB, Corle D, Hess TM, Woloj GM, Boisserie F, Anderson WF, Viner JL, Bagheri D, Burn J, Chung DC, Dewar T, Foley TR, Hoffman N, Macrae F, Pruitt RE, Saltzman JR, Salzberg B, Sylwestrowicz T, Gordon GB, Hawk ET, APC Study Investigators** 2006 Celecoxib for the prevention of sporadic colorectal adenomas. N Engl J Med 355:873-884

316. **Farley JH, Truong V, Goo E, Uyehara C, Belnap C, Larsen WI** 2006 A randomized double-blind placebo-controlled phase II trial of the cyclooxygenase-2 inhibitor Celecoxib in the treatment of cervical dysplasia. Gynecol Oncol 103:425-430

317. **Moore A, Chen CL, Davis JR, Morris ID** 1993 Insulin-like growth factor-I mRNA expression in the interstitial cells of the rat testis. J Mol Endocrinol 11:319-324

318. **Sriraman V, Rao VS, Sairam MR, Rao AJ** 2000 Effect of deprival of LH on Leydig cell proliferation: involvement of PCNA, cyclin D3 and IGF-1. Molecular and Cellular Endocrinology 162:113-120

Insulin-Like Growth Factor-I, Regulating Aromatase Expression through Steroidogenic Factor 1, Supports Estrogen-Dependent Tumor Leydig Cell Proliferation

Rosa Sirianni,[1] Adele Chimento,[1] Rocco Malivindi,[1] Ignazio Mazzitelli,[1] Sebastiano Andò,[2] and Vincenzo Pezzi[1]

Departments of [1]Pharmaco-Biology and [2]Cell Biology, University of Calabria, Arcavacata di Rende, Cosenza, Italy

Abstract

The aim of this study was to investigate the role of estrogens in Leydig cell tumor proliferation. We used R2C rat Leydig tumor cells and testicular samples from Fischer rats with a developed Leydig tumor. Both experimental models express high levels of aromatase and estrogen receptor α (ERα). Treatment with exogenous 17β-estradiol (E_2) induced proliferation of R2C cells and up-regulation of cell cycle regulators cyclin D1 and cyclin E, the expression of which was blocked by addition of antiestrogens. These observations led us to hypothesize an E_2/ERα–dependent mechanism for Leydig cell tumor proliferation. In determining the molecular mechanism responsible for aromatase overexpression, we found that total and phosphorylated levels of transcription factors cyclic AMP–responsive element binding protein and steroidogenic factor 1 (SF-1) were higher in tumor samples. Moreover, we found that tumor Leydig cells produce high levels of insulin-like growth factor I (IGF-I), which increased aromatase mRNA, protein, and activity as a consequence of increased total and phosphorylated SF-1 levels. Specific inhibitors of IGF-I receptor, protein kinase C, and phosphatidylinositol 3-kinase determined a reduction in SF-1 expression and in IGF-I–dependent SF-1 recruitment to the aromatase PII promoter. The same inhibitors also inhibited aromatase expression and activity and, consequently, R2C cell proliferation. We can conclude that one of the molecular mechanisms determining Leydig cell tumorigenesis is an excessive estrogen production that stimulates a short autocrine loop determining cell proliferation. In addition, cell-produced IGF-I amplifies estrogen signaling through an SF-1–dependent up-regulation of aromatase expression. The identification of this molecular mechanism will be helpful in defining new therapeutic approaches for Leydig cell tumors. [Cancer Res 2007;67(17):8368–77]

Introduction

The etiology and pathogenesis of human testicular tumors are poorly defined. It has been reported that serum estrogen levels are elevated in patients with testicular germ cell cancer as a consequence of increased local estrogen production reflecting higher aromatase activity present in Sertoli and Leydig cells (1). Ninety-five percent of all human testicular neoplasms arise from germinal cells whereas Leydig cell tumors are the most common tumors of the gonadal stroma (2).

In rodents, reproductive system tumors are uncommon in general, with the few exceptions of Leydig cell and ventral prostatic neoplasms in some rat strains (3) or non-inbred mice (4); however, analogously to the human (5), chronic administration of estrogens induces testicular tumors.

A useful model used to investigate whether excess estrogens might have a central role in the mechanism leading to testicular tumorigenesis are transgenic mice overexpressing aromatase and presenting enhancement of circulating 17β-estradiol (E_2) levels (6). About half of the male mice are infertile and/or have enlarged testis and show Leydig cell hyperplasia and Leydig cell tumors (6), whereas the female mice reveal mammary gland hyperplasia associated with an altered expression pattern of proteins involved in apoptosis, cell cycle, growth, and tumor suppression (7). Whereas the effects of estrogen on mammary gland tumorigenesis in human and rodents are well known, the role of aromatase overexpression and *in situ* estrogen production in testicular tumorigenesis has not been clearly defined. In this study, we have investigated the molecular mechanisms causing aromatase overexpression and the effect of estradiol (E_2) overproduction on rat Leydig cell tumor proliferation. As an experimental model, we used the R2C rat Leydig tumor cell line; to validate our *in vitro* data in an *in vivo* model, we used Leydig cell tumors from older Fisher rats characterized by an exceptionally high incidence of spontaneous neoplasm with aging (8).

Aromatase activity is regulated primarily at the level of gene expression and is present in testicular somatic cells and along the maturative phases of male germ cells (9, 10). The *CYP19* gene that encodes aromatase has at least eight unique promoters that are used in a tissue-specific manner (11). The proximal promoter II regulates aromatase expression in human fetal and adult testis, R2C and H540 rat Leydig tumor cells, and purified preparations of rat Leydig, Sertoli, and germ cells (12, 13). Specific sequences seem to be mainly involved in aromatase expression: a sequence that contains a half-site binding nuclear receptors (AGGTCA) in position −90 in the rat binding steroidogenic factor 1 (SF-1; ref. 14) and cyclic AMP (cAMP)–responsive element (CRE)–like sequences binding cAMP-responsive element binding protein (CREB)/activating transcription factor protein family members (15, 16) localized upstream at a more distal position, in the rat in positions −169 (TGCACGTCA), −335 (TGAACTCA), and −231 (TGAAATCA; ref. 17). Similar responsive elements (binding CRE and SF-1) have been reported for the steroidogenic acute regulatory (StAR) protein gene promoter (18) whose expression is regulated by insulin-like growth factor-I (IGF-I) signaling in Leydig cells. Because the StAR protein is involved in the transfer of cholesterol from the outer to the inner

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

R. Sirianni and A. Chimento contributed equally to this work.

Requests for reprints: Vincenzo Pezzi, Department of Pharmaco-Biology, Università della Calabria, 87036 Rende, Cosenza, Italy. Phone: 39-0984-493157; Fax: 39-0984-493271; E-mail: v.pezzi@unical.it.

doi:10.1158/0008-5472.CAN-06-4064

mitochondrial membrane, the rate-limiting and regulated step in steroidogenesis IGF-I plays an important role in the regulation of testicular steroid biosynthesis.

For these reasons, we investigated the role of IGF-I, a peptide also shown to have a role in testicular growth and development and in the control of Leydig cell number (19). IGF-I is produced locally in the testis, in Sertoli, Leydig, and peritubular cells derived from the immature rat testis and cultured *in vitro* (20, 21). The crucial role of IGF-I in the development and function of Leydig cells was highlighted by studies on *IGF-I* gene knockout mice (22, 23). The failure of adult Leydig cells to mature and the reduced capacity for testosterone production are caused by deregulated expression of testosterone biosynthetic and metabolizing enzymes (24). Expression levels of all mRNA species associated with testosterone biosynthesis are lower in the absence of IGF-I. However, this study did not investigate the effect of IGF-I on aromatase expression, although an effect could be supposed.

Starting from these findings, in this study we investigated whether a testicular overproduction of IGF-I could be one of the mechanisms determining aromatase overexpression in rat tumor Leydig cells through the activation of specific transcription factors. The elevated aromatase-dependent E_2 production in Leydig cells, through an autocrine/paracrine mechanism mediated by their own receptors, could contribute to the hormone dependence of testicular tumorigenesis by stimulating Leydig tumor cell proliferation.

Materials and Methods

Cell cultures and animals. TM3 cells (immature mouse Leydig cell line) were cultured in DMEM/F-12 supplemented with 5% horse serum (HS), 2.5% fetal bovine serum (FBS), and antibiotics (Invitrogen S.R.L.); R2C cells (rat Leydig tumor cell line) were cultured in Ham/F-10 supplemented with 15% HS, 2.5% FBS, and antibiotics (Invitrogen). Male Fischer 344 rats (a generous gift of Sigma-Tau), 6 (FRN) and 24 (FRT) months of age, were used for studies. Twenty-four-month-old animals presented spontaneously developed Leydig cell tumors, which were absent in younger animals. Testes of all animals were surgically removed by qualified, specialized animal care staff in accordance with the Guide for Care and Use of Laboratory Animals (NIH) and used for experiments.

Aromatase activity assay. The aromatase activity in subconfluent R2C cell culture medium was measured by tritiated water-release assay using 0.5 μmol/L [1β-^{3}H(N)]androst-4-ene-3,17-dione (DuPont NEN) as a substrate (25). Incubations were done at 37°C for 2 h under a 95%:5% air/CO_2 atmosphere. Obtained results were expressed as picomoles per hour (pmol/h) and normalized to milligrams of protein (pmol/h/mg protein).

RIA. Before the experiments, TM3 cells were maintained overnight in DMEM/F-12 and R2C cells in Ham/F-10 (medium only). The estradiol content of medium recovered from each well was determined against standards prepared in low-serum medium using a RIA kit (DSL 43100; Diagnostic System Laboratories). Results of the assay were normalized to the cellular protein content per well and expressed as picomoles per milligram of cell protein.

To measure IGF-I concentration in testicular extracts, testes were weighed, homogenated in 500 μL of 0.05 mol/L Tris/HCl (pH 7.6) plus protease inhibitors, and then submitted to ultrasonication followed by centrifugation, as previously published (26). IGF-I content in testicular extracts and in medium recovered from each well of R2C and TM3 cells was determined following extraction and assay protocols provided with the mouse/rat IGF-I RIA kit (DSL 2900; Diagnostic System Laboratories).

Chromatin immunoprecipitation. This assay was done using the chromatin immunoprecipitation assay kit from Upstate with minor modifications in the protocol. R2C cells were grown in 100-mm plates. Confluent cultures (90%) were treated for 24 h with AG1024 (Sigma), PD98059 (Calbiochem, VWR International S.R.L.), LY294002 (Calbiochem, VWR International), GF109203X (Calbiochem, VWR International), or for increasing times with 100 ng/mL IGF-I (Sigma), or left untreated. Following treatment, DNA/protein complexes were cross-linked with 1% formaldehyde at 37°C for 10 min. Next, cells were collected and resuspended in 400 μL of SDS lysis buffer (Upstate Technology) and left on ice for 10 min. Then, cells were sonicated four times for 10 s at 30% of maximal power and collected by centrifugation at 4°C for 10 min at 14,000 rpm. Ten microliters of the supernatants were kept as input (starting material, to normalize results) whereas 100 μL were diluted 1:10 in 900 μL of chromatin immunoprecipitation dilution buffer (Upstate Technology) and immunocleared with 80 μL of sonicated salmon sperm DNA/protein A agarose (Upstate) for 6 h at 4°C. Immunocomplex was formed using 1 μL of 1:5 dilution of specific anti–SF-1 antibody (provided by Prof. Ken-ichirou Morohashi, Division for Sex Differentiation, National Institute for Basic Biology, National Institutes of Natural Sciences, Myodaiji-cho, Okazaki, Japan) overnight at 4°C. Immunoprecipitation with salmon sperm DNA/protein A agarose was continued at 4°C until the following day. DNA/protein complexes were reverse cross-linked overnight at 65°C. Extracted DNA was resuspended in 20 μL of Tris-EDTA buffer. A 3-μL volume of each sample and input was used for PCR using CYP19 promoter II–specific primers. The PCR conditions were 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C for 30 cycles using the following primers: forward, 5′-TCAAGGGTAGGAATTGGGAC-3′; reverse, 5′-GGTGCTGGAATGGACAGATG-3′. Amplification products were analyzed on a 1% agarose gel and visualized by ethidium bromide staining. In control samples, nonimmune rabbit immunoglobulin G was used instead of specific antibodies.

Real-time reverse transcription-PCR. Before the experiments, cells were maintained overnight in low-serum medium. Cells were then treated for the indicated times and RNA was extracted from cells using the TRizol RNA isolation system (Invitrogen). TRizol was also used to homogenize total tissue of normal (FRNT) and tumor (FRTT) Fisher rat testes for RNA extraction. Each RNA sample was treated with DNase I (Ambion), and purity and integrity of the RNA were confirmed spectroscopically and by gel electrophoresis before use. One microgram of total RNA was reverse transcribed in a final volume of 30 μL using the ImProm-II Reverse transcription system kit (Promega, Promega Italia S.R.L.); cDNA was diluted 1:3 in nuclease-free water, aliquoted, and stored at −20°C. Primers for the amplification were based on published sequences for the rat *CYP19*, rat *CREB*, and rat *SF-1* genes. The nucleotide sequences of the primers for *CYP19* were forward, 5′-GAGAAACTGGAAGACTGTATGGAT-3′, and reverse, 5′-ACTGATTCACGTTCTCCTTTGTCA-3′. For CREB amplification, we used the following primers: forward, 5′-AATATGCACAGACCACTGATGGA-3′, and reverse, 5′-TGCTGTGCGAATCTGGTATGTT-3′; for SF-1 amplification, primers have been previously published (27). PCR reactions were done in the iCycler iQ Detection System (Bio-Rad) using 0.1 μmol/L of each primer, in a total volume of 30-μL reaction mixture following the manufacturer's recommendations. SYBR Green Universal PCR Master Mix (Bio-Rad) with the dissociation protocol was used for gene amplification; negative controls contained water instead of first-strand cDNA. Each sample was normalized on the basis of its 18S rRNA content. The 18S quantification was done using a TaqMan rRNA Reagent kit (Applied Biosystems) following the method provided in the TaqMan rRNA Control Reagent kit (Applied Biosystems). The relative gene expression levels were normalized to a calibrator that was chosen to be the basal, untreated sample. Final results were expressed as *n*-fold differences in gene expression relative to 18S rRNA and calibrator, calculated using the $\Delta\Delta C_t$ method as follows:

$$n - \text{fold} = 2^{-(\Delta C_{\text{tsample}} - \Delta C_{\text{tcalibrator}})}$$

where ΔC_t values of the sample and calibrator were determined by subtracting the average C_t value of the 18S rRNA reference gene from the average C_t value of the different genes analyzed.

Western blot analysis. R2C and TM3 cells or total tissue of FRNT and FRTT were lysed in ice-cold radioimmunoprecipitation assay buffer containing protease inhibitors (20 mmol/L Tris, 150 mmol/L NaCl, 1% Igepal, 0.5% sodium deoxycholate, 1 mmol/L EDTA, 0.1% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, 0.15 units/mL aprotinin, and 10 μmol/L leupeptin) for protein extraction. The protein content was determined by the Bradford method. The proteins were separated on 11% SDS-polyacrylamide gel and then electroblotted onto a nitrocellulose membrane. Blots were incubated overnight at 4°C with (*a*) antihuman *P*450 aromatase antibody (1:50; Serotec, MCA 2077), (*b*) anti-ERα (F-10) antibody (1:500; Santa Cruz Biotechnology), (*c*) anti-ERβ (H-150) antibody (1:1,000; Santa Cruz Biotechnology), (*d*) anti–cyclin D1 (M-20) antibody (1:1,000; Santa Cruz Biotechnology), (*e*) anti–cyclin E (M-20) antibody (1:1,000; Santa Cruz Biotechnology), (*f*) anti-CREB antibodies [48H2 (1:1,000; Cell Signaling Technology) and AHO0842 (1:1,000; Biosource, Inc.)]; (*g*) anti-pCREB Ser^{133} (87G3; 1:1,000; Cell Signaling Technology) or anti-pCREB $Ser^{129/133}$ (1:1,000; Biosource, Inc.), (*h*) anti–SF-1 (1:1,000; provided by Prof. Ken-ichirou Morohashi), (*i*) anti–pSF-1 (1:1,000; provided by Dr. Holly A. Ingraham, Department of Physiology, University of California, San Francisco, San Francisco, California), (*j*) anti-actin (C-2) antibody (1:1,000; Santa Cruz Biotechnology), and (*k*) anti–IGF-I receptor (IGF-IR; 1:800; Santa Cruz Biotechnology). Membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (Amersham Pharmacia Biotech) and immunoreactive bands were visualized with the enhanced chemiluminescence Western blotting detection system (Amersham Biosciences). To ensure equal loading of proteins, membranes were stripped and incubated overnight with β-actin antiserum.

Cell proliferation assay. For proliferative analysis, a total of 1×10^5 cells were seeded onto 12-well plates in complete medium and allowed to grow for 2 days. Before the experiments, cells were maintained overnight in Ham/F-10 medium and were treated the next day with ICI 182780 (a gift from Astra-Zeneca), 4-hydroxytamoxifen (Sigma), and letrozole (a gift from Novartis Pharma AG) and E_2 (Sigma), or treated for 24 h with IGF-I alone or in combination with inhibitors, or incubated with an anti–IGF-I antibody (Santa Cruz Biotechnology). Control (basal) cells were treated with the same amount of vehicle alone (DMSO) that never exceeded the concentration of 0.01% (v/v). [^{3}H]Thymidine incorporation was evaluated after a 24-h incubation period with 1 μCi of [^{3}H]thymidine (Perkin-Elmer

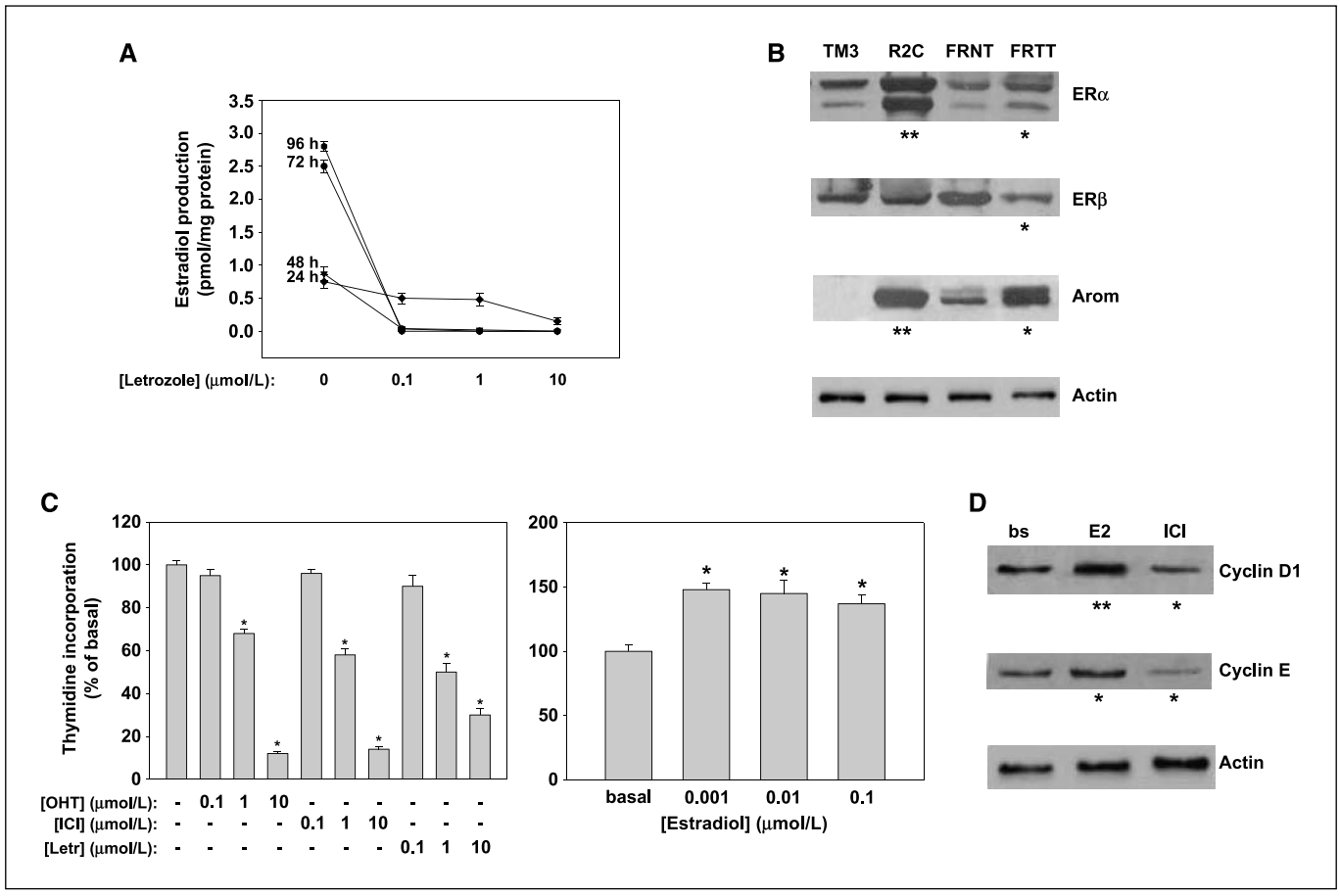


Figure 1. E_2 production and autocrine effects in R2C cells. *A,* cells were treated for the indicated times in HAM-F10 in the absence (0) or presence of aromatase inhibitor letrozole (0.1, 1, and 10 μmol/L). Every 24 h, before renewing treatment, cell culture medium was removed and analyzed for steroid content. E_2 content in R2C culture medium was determined by RIA and normalized to the cell culture well protein content. *Points,* mean from three separate cell culture wells; *bars,* SE. *B,* Western blot analyses of ERα, ERβ, and aromatase (*Arom*) were done on 50 μg of total proteins extracted from TM3 and R2C cells or from tissues of normal (FRNT) and tumor (FRTT) Fisher rat testes. Representative of three independent experiments. β-Actin was used as a loading control. *C,* R2C cell proliferation was evaluated by [^{3}H]thymidine incorporation analysis. Cells were treated for 96 h in HAM-F10 in the absence (−) or presence of antiestrogens hydroxytamoxifen (*OHT*) or ICI 182780 (*ICI*) or aromatase inhibitor letrozole (*Letr*) at the indicated concentrations or treated with estradiol (0.001–0.1 μmol/L) for 24 h after being cultured for 48 h in serum-free HAM-F10, removing and renewing cell culture medium every 24 h. *D,* R2C cells were cultured for 48 h in serum-free HAM-F10; every 24 h, cell culture medium was removed and renewed. Cells were then treated for 24 h in the absence (basal, *bs*) or presence of estradiol (1 nmol/L) and ICI 182780 (1 μmol/L) before extracting total proteins. Western blot analysis of cyclin D1 and cyclin E was done on 50 μg of total proteins extracted from R2C cells. β-Actin was used as a loading control. Protein expression in each lane was normalized to the β-actin content and expressed as fold over control represented by normal cells (*B*) or basal condition (*D*). Normalized absorbances were subjected to statistical analysis; statistically significant differences are indicated (*, $P < 0.05$; **, $P < 0.01$, compared with basal or control).

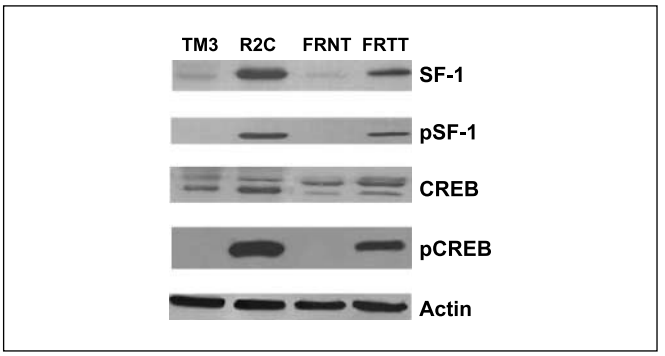


Figure 2. Expression of total and phosphorylated forms of SF-1 and CREB. Western blot analyses of SF-1, pSF-1, CREB, and pCREB were done on 50 μg of total proteins extracted from TM3 and R2C cells or from tissues of normal (FRNT) and tumor (FRTT) Fisher rat testes. Representative of three independent experiments with similar results. β-Actin was used as a loading control. Protein expression in each lane was normalized to the β-actin content.

Life Sciences) per well. Cells were washed once with 10% trichloroacetic acid, twice with 5% trichloroacetic acid, and lysed in 1 mL of 0.1 mol/L NaOH at 37°C for 30 min. The total suspension was added to 10 mL optifluor fluid and was counted in a scintillation counter.

Data analysis and statistical methods. Pooled results from triplicate experiments were analyzed by one-way ANOVA with Student-Newman-Keuls multiple comparison methods, using SigmaStat version 3.0 (SPSS).

Results

Estradiol induces Leydig cell tumor proliferation through an autocrine mechanism. We carried out our study using R2C Leydig tumor cells as model system. These cells have been shown to have high aromatase expression and activity (14). We also used another Leydig cell line, TM3, as a normal control and analyzed testes from older and younger Fischer rats. Aged animals have a high incidence of spontaneous Leydig cell neoplasm (8, 28), a phenomenon not observed in younger animals, allowing us to use them as a good *in vivo* model to confirm results obtained in cell lines. Our first step was to measure estradiol content in culture media of R2C and TM3 cells. Whereas E_2 levels in TM3 medium were extremely low (data not shown) in R2C cells, E_2 levels after 24 h were 0.5 pmol/mg protein and increased by 7-fold at 96 h (Fig. 1*A*). This production was dependent on high constitutive active aromatase activity because the presence of the aromatase inhibitor letrozole was able to decrease E_2 production at all doses and times tested (Fig. 1*A*). E_2 levels after 24-h treatment with letrozole were still detectable but were completely knocked down when we removed the medium after 24 h and renewed the treatment for an additional 24 h. The same effect was maintained at the other two time points investigated (Fig. 1*A*). Once estradiol is produced, it can exert its actions by binding to specific receptors, the estrogen receptors α and β (ERα and ERβ). Analysis of the two protein receptor isoforms in our models showed that tumor Leydig cells express both isoforms of ER (Fig. 1*B*). Particularly, the α isoform seems to be more expressed in R2C cells with respect to TM3 and in FRTT with respect to its control FRNT (Fig. 1*B*) in which ERβ, instead, is more expressed (Fig. 1*B*). In R2C as well as in FRTT, an increase in the ERα/ERβ ratio was observed (Supplementary Fig. S1*A*). Moreover, aromatase protein content is extremely high in tumor samples (Fig. 1*B*).

Our next experiments showed that ERs are required for proliferation through a short autocrine loop maintained by endogenous E_2 production in Leydig tumor cells. For instance, the use of both antiestrogens 4-hydroxytamoxifen and ICI 182780 and the use of the aromatase inhibitor letrozole determined a dose-dependent inhibition of cell proliferation (Fig. 1*C*). Among the different doses tested, the highest dose of 4-hydroxytamoxifen (10 μmol/L) was able to inhibit cell proliferation by 90%; 10 μmol/L ICI 182780, by 86%; and 10 μmol/L letrozole, by 70%. Moreover,

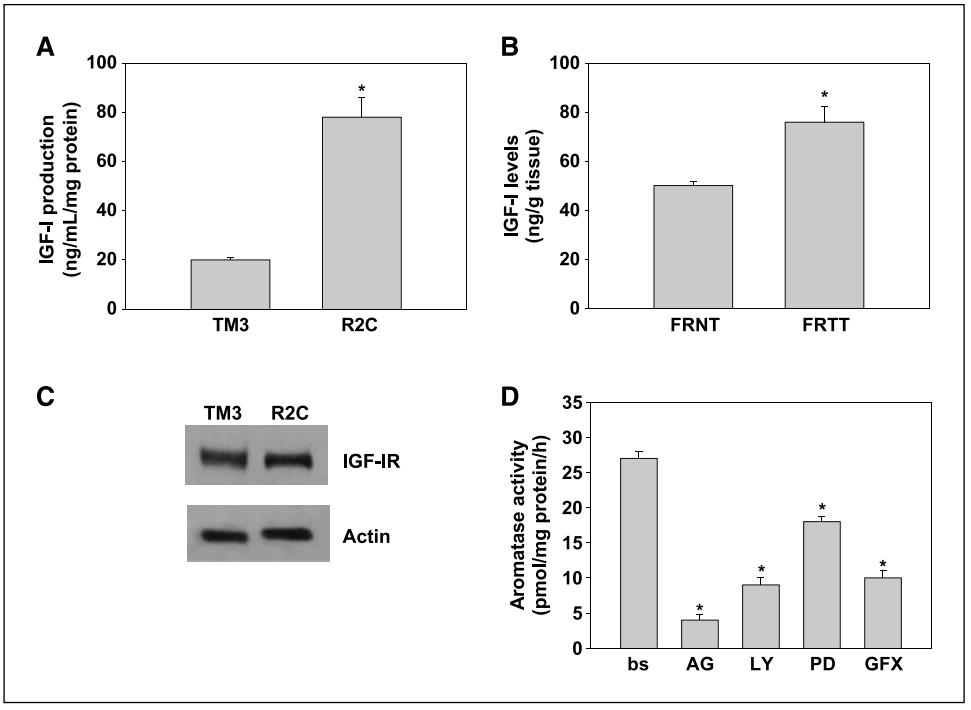


Figure 3. IGF-I production and autocrine effects in Leydig cells. *A,* TM3 and R2C cells were cultured for 24 h in serum-free medium and IGF-I levels in culture medium were determined by RIA. IGF-I levels were normalized to the cell culture well protein content. *Columns,* mean of three independent experiments each done in triplicate; *bars,* SE. *B,* total protein extracts from FRNT and FRTT were assayed for IGF-I content. IGF-I levels were normalized to the tissue weight. *Columns,* mean of three independent samples; *bars,* SE. *, $P < 0.01$, compared with control conditions, represented by TM3 cells or FRNT. *C,* Western blot analysis of IGF-IR in TM3 and R2C cells. β-Actin was used as a loading control. *D,* cells were treated with AG1024 (*AG*; 20 μmol/L), LY294002 (*LY*; 10 μmol/L), PD98059 (*PD*; 20 μmol/L), and GF109203X (*GFX*; 20 μmol/L). Aromatase activity was assessed by using the modified tritiated water method. Results obtained are expressed as picomoles of $[^3H]H_2O$ released per hour and normalized to the well protein content (pmol/h/mg protein). *Columns,* mean of three independent experiments each done in triplicate; *bars,* SE. *, $P < 0.01$, compared with basal.

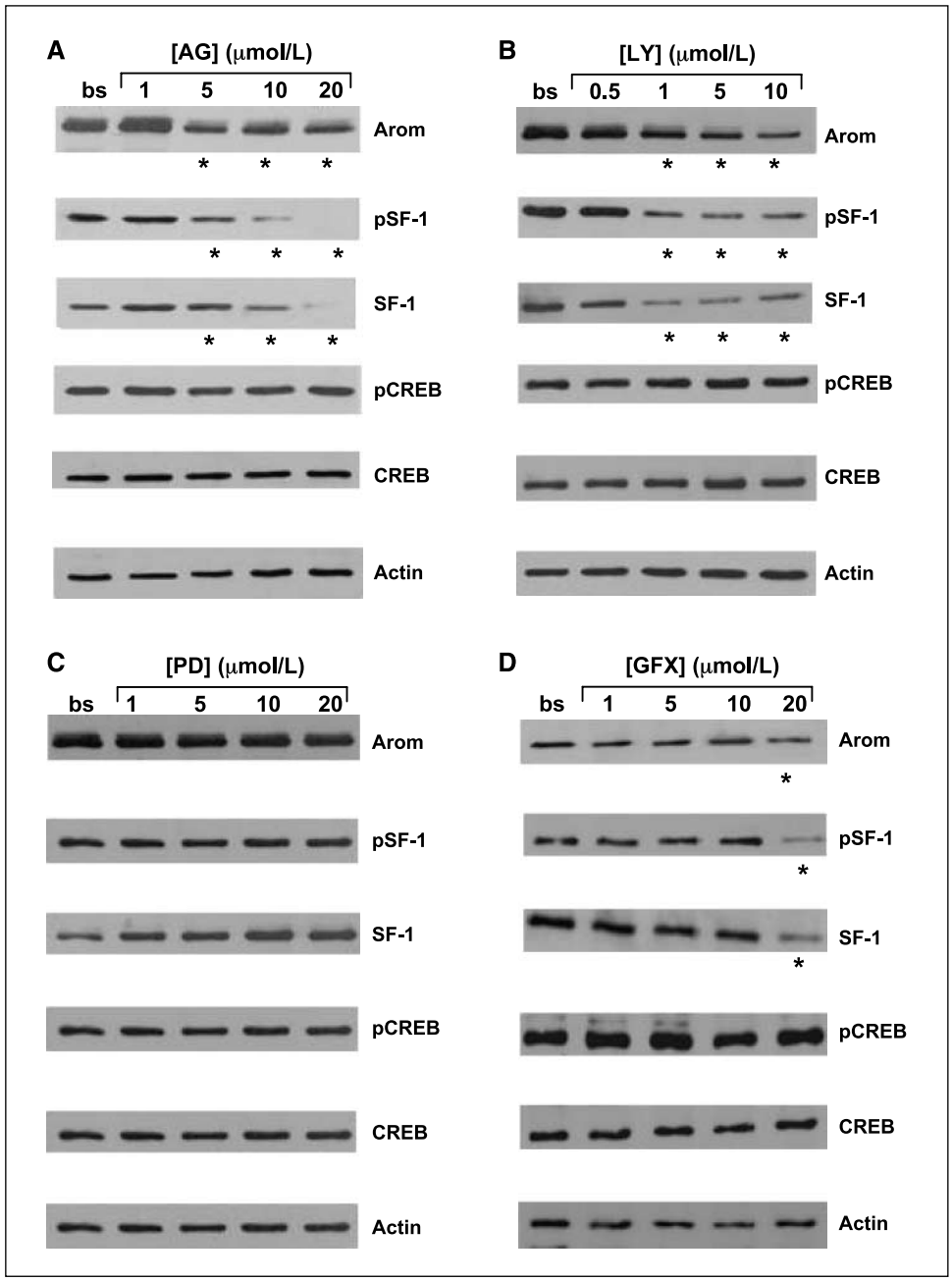


Figure 4. Effects of IGF-I pathway inhibitors on aromatase, SF-1, and CREB expression in R2C cells. *A* to *D,* Western blot analyses were done on 50 μg of total proteins extracted from R2C cells untreated (*bs*) or treated for 24 h with the indicated doses of AG1024 (*A*), LY294002 (*B*), PD98059 (*C*), and GF109203X (*D*). Representative of three independent experiments with similar results. β-Actin was used as a loading control. Normalized absorbances were subjected to statistical analysis; statistically significant differences are indicated (*, $P < 0.01$, compared with basal).

after starving cells for a prolonged time and changing the medium everyday to remove local E_2 production, we found that addition of 1, 10, and 100 nmol/L E_2 stimulated Leydig tumor cell proliferation (Fig. 1*C*), overcoming the inhibition induced by letrozole (Supplementary Fig. S1*B*). The stimulatory effect of E_2 was concomitant with increased levels of cell cycle regulators cyclin D1 and cyclin E, whose expression was inhibited by the pure antiestrogen ICI 182780 (Fig. 1*D*). All these results address how the classic E_2/ERα signaling may control Leydig cell tumor growth and proliferation similarly to what was observed in other estrogen-dependent tumors.

Aromatase overexpression is determined by constitutive activation of transcription factors SF-1 and CREB. Aromatase gene transcription in rat Leydig cells is driven by the PII promoter, which is mainly regulated through three CRE-like sites and one NRE site binding SF-1 and LRH-1 (14, 27). Constitutively active levels of CREB have previously been shown in R2C cells (29). Here, we confirmed these data and showed that FRTT have a high phosphorylated CREB status (Fig. 2), together with enhanced expression and phosphorylation of SF-1, with respect to FRNT (Fig. 2). Furthermore, we showed the presence of high expression levels of SF-1 with the phosporylated protein present in R2C but not in TM3 cells (Fig. 2).

IGF-I is produced by R2C cells and induces aromatase expression through phosphatidylinositol 3-kinase– and protein kinase C–mediated activation of SF-1. Starting from previous findings showing the ability of IGF-I to activate SF-1 and CREB, which leads to an increase in StAR transcription and then

steroidogenesis (18), we investigated the role of this factor in regulating aromatase. Determination of IGF-I content in TM3 and R2C culture medium by RIA revealed a significant difference in growth factor production, with R2C cells producing ~4-fold higher IGF-I amounts (Fig. 3*A*). Moreover, we measured IGF-I content in testicular tissues, revealing a significant difference between FRTT and FRNT (Fig. 3*B*). IGF-I exerts its actions by binding to specific receptors (IGF-IR); however, we did not reveal differences in IGF-IR expression between TM3 and R2C cells (Fig. 3*C*).

On binding to IGF-IR, IGF-I activates three major transductional pathways: Ras/Raf/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/AKT, and phospholipase C (PLC)/protein kinase C (PKC). To show the involvement of IGF-I transductional pathways in modulating aromatase expression in Leydig cell tumors, we used specific inhibitors of IGF-IR (AG1024), extracellular signal–regulated kinase (ERK)-1/2 (PD98059), PI3K (LY294002), and PKC (GF109203X). The IGF-IR inhibitor was able to inhibit aromatase activity by 85%; LY294002 determined 65% inhibition; PD98059, 35%; and GF109203X, 61% (Fig. 3*D*). A similar inhibitory pattern, except for PD98059, was observed also on aromatase mRNA (Supplementary Fig. S2*A*) and protein content (Fig. 4*A*–*D*). All of the different inhibitors, excluding PD98059, were able to reduce SF-1 mRNA (Supplementary Fig. S2*B*), whereas CREB remained unchanged (Supplementary Fig. S2*C*). Analysis of protein levels by Western blot confirmed the data from mRNA (Fig. 4*A*–*D*). Treatments with increasing doses of AG1024, LY294002, and GF109203X, but not PD98059, were able to induce a dose-dependent inhibition of total and phosphorylated levels of SF-1 without affecting CREB (Fig. 4*A*–*D*).

Addition of exogenous amounts of IGF-I was able to induce a significant increase of 2- and 3.8-fold in aromatase mRNA at 12 and 24 h, respectively (Fig. 5*A*). Aromatase protein levels under the same treatments reflected the mRNA data (Fig. 5*B*). Analysis of expression levels of total and phosphorylated forms of transcription factors SF-1 and CREB showed an increase in SF-1 and pSF-1 in the presence of IGF-I starting at 4 h, whereas no differences were observed for CREB at any of the investigated times (Fig. 5*B*). AG1024, LY294002, and GF109203X were able to inhibit IGF-I effects on CYP19 mRNA and protein levels (Fig. 5*C* and *D*) as a consequence of a decreased SF-1 expression (Fig. 5*D*).

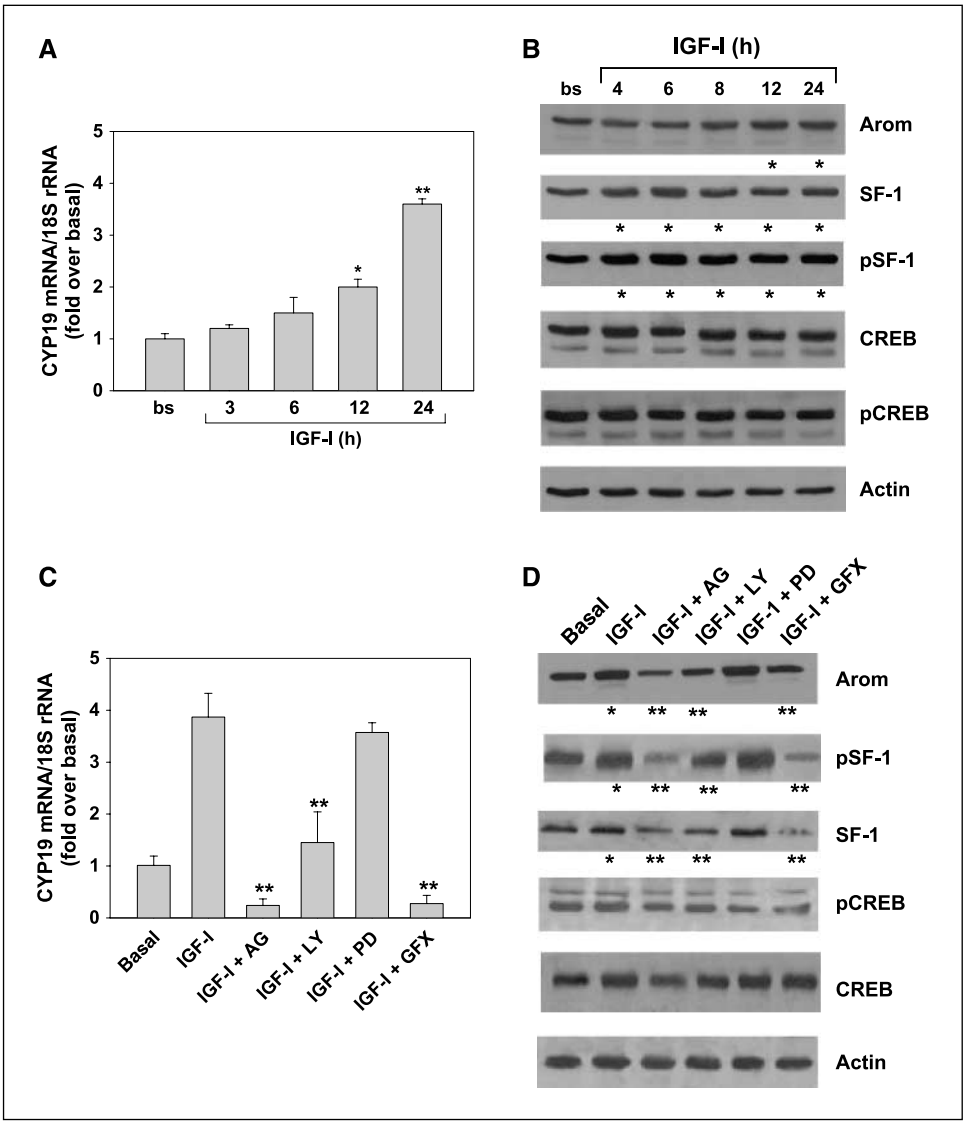


Figure 5. Effects of IGF-I on aromatase, SF-1, and CREB expression in R2C cells. Cells were treated in serum-free medium for the indicated times with IGF-I (100 ng/mL) or for 24 h with AG1024 (20 μmol/L), LY294002 (10 μmol/L), PD98059 (20 μmol/L), and GF109203X (20 μmol/L), alone or in combination with IGF-I (100 ng/mL). *A* and *C,* total RNA was extracted from R2C cells untreated or treated as indicated. Real-time reverse transcription-PCR was used to analyze CYP19 mRNA levels. *Columns,* mean of values from three separate RNA samples; *bars,* SE. Each sample was normalized to its 18S rRNA content. *, $P < 0.01$; **, $P < 0.001$, compared with basal. *B* and *D,* Western blot analyses were done on 50 μg of total proteins extracted from R2C cells untreated (basal) or treated as indicated. Representative of three independent experiments with similar results. β-Actin was used as a loading control. Normalized absorbances were subjected to statistical analysis; statistically significant differences are indicated (*, $P < 0.01$, compared with basal; **, $P < 0.01$, compared with IGF-I).

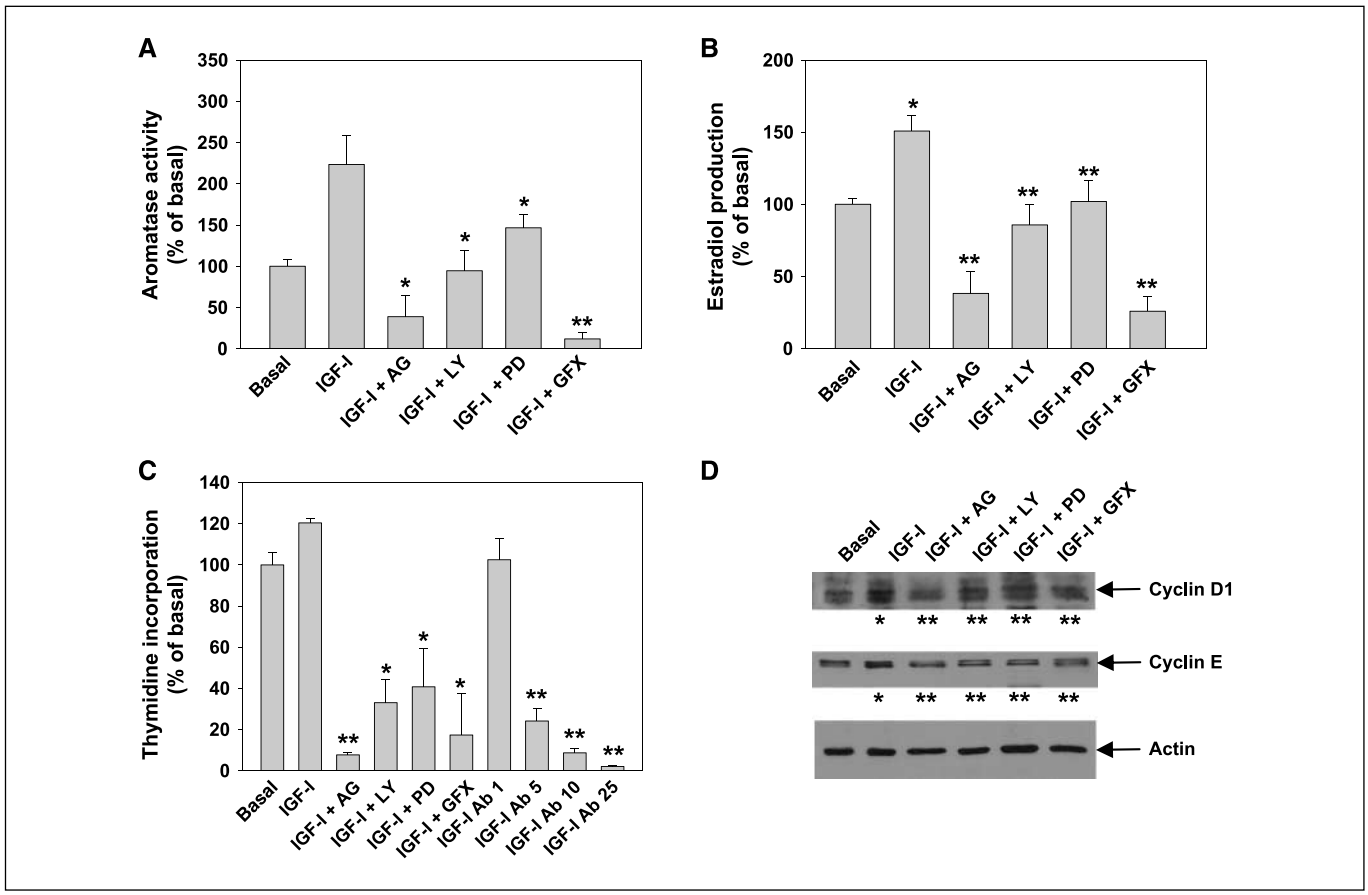


Figure 6. Effects of IGF-I and IGF-I pathway inhibitors on estradiol production and R2C cell proliferation. Cells were treated in serum-free medium for 24 h with IGF-I (100 ng/mL) alone or in combination with AG1024 (20 μmol/L), LY294002 (10 μmol/L), PD98059 (20 μmol/L), and GF109203X (20 μmol/L). *A,* aromatase activity is expressed as percent of basal. *Columns,* mean of three independent experiments each done in triplicate; *bars,* SE. *, $P < 0.01$; **, $P < 0.001$, compared with IGF-I. *B,* R2C cells were maintained for 48 h in serum-free medium, before being treated for 24 h. E_2 content in R2C culture medium was determined by RIA and normalized to the cell culture well protein content. *Columns,* mean percent of basal; *bars,* SE. *, $P < 0.05$, compared with basal; **, $P < 0.01$, compared with IGF-I. *C,* R2C cell proliferation was evaluated by [^{3}H]thymidine incorporation analysis. Cells were maintained for 24 h in serum-free medium and treated for 24 h as indicated. IGF-I antibody (*IGF-I Ab*) was added to the medium at 1, 5, 10, and 25 μg/mL. *Columns,* mean percent of untreated (basal) cells (100%) from three independent experiments each done in triplicate; *bars,* SE. *, $P < 0.01$; **, $P < 0.001$, compared with IGF-I. *D,* Western blot analyses were done on 50 μg of total proteins extracted from R2C cells treated as indicated. Representative of three independent experiments with similar results. β-Actin was used as a loading control. Normalized absorbances were subjected to statistical analysis; statistically significant differences are indicated (*, $P < 0.01$, compared with basal; **, $P < 0.01$, compared with IGF-I).

We also carried out chromatin immunoprecipitation assays to investigate how IGF-I influences binding of transcription factors to the aromatase PII promoter. The increase in SF-1 protein content that was seen under IGF-I treatment reflected an increase in SF-1 binding to the PII promoter (Supplementary Fig. S3*A*). Moreover, we evidenced how in basal conditions (Supplementary Fig. S3*B*), as well as after IGF-I treatment (Supplementary Fig. S3*C*), all the different inhibitors, but not PD98059, reduced SF-1 binding.

IGF-I–induced estradiol production modulates R2C cell proliferation. Treatment with IGF-I induces aromatase activity and estradiol production, which are decreased by AG1024, LY294002, and GF109203X, as well as by PD98059 (Fig. 6*A* and *B*). The observed changes in estradiol production modified R2C cell proliferative behavior (Fig. 6*C*). In addition, the use of an anti–IGF-I antibody in immunoneutralization experiments caused a dose-dependent inhibition in tritiated thymidine incorporation (Fig. 6*C*). The ability of IGF-I to stimulate, and that of the inhibitors to block, cell proliferation was linked to an alteration in cyclin D1 and cyclin E expression (Fig. 6*D*).

Discussion

The current study aimed to explain the molecular mechanism responsible for aromatase overexpression in tumor Leydig cells leading to a consequent excess of *in situ* estradiol production that sustains tumor cell growth and proliferation.

The mammalian testis is capable of estrogen synthesis, which is regulated by different factors at different ages. In mature animals, aromatization of testosterone to estradiol is enhanced by luteinizing hormone (LH)/chorionic gonadotropin (CG) and not by follicle-stimulating hormone. The site of this synthesis seems to be age dependent, at least in some species such as the rat (30). Leydig cells are an elective target site of LH/CG that controls testosterone biosynthesis as well as its conversion to estradiol through aromatase activity. Alterations in local estrogen synthesis may have significant consequences in malignancy of these cells. In the present study, we observed that R2C cells release a conspicuous amount of E_2 from cellular storage in a time-dependent manner. In this condition, E_2 production (1,300 ± 230 pg/10^6 cells/24 h) is significantly higher compared with E_2 levels produced by TM3 cells and by primary rat Leydig cell cultures (246 pg/10^6

3β-hydroxysteroid dehydrogenase–positive cells/24 h; ref. 31). E_2 synthesis by R2C cells was abrogated by treatment with letrozole, addressing how steroid production is dependent on high constitutive aromatase activity. A strong increase in aromatase expression was observed in R2C cells compared with the normal cell line TM3 control, as well as in FRTT compared with FRNT. These findings agree with a previous study on human tissues showing that the increase in estrogen synthesis, as a consequence of a more intense aromatase activity, is higher in the Leydig cell tumor fraction than in normal tissues surrounding the tumor of the same patient (32).

Mediators of the physiologic effects of estrogens are ERα and ERβ. An enhanced expression of ERα, resulting in an increased ERα/ERβ ratio, was observed in R2C compared with TM3 cell line, as well as in FRTT compared with FRNT. This is in agreement with previous reports showing that transgenic mice overexpressing aromatase have an enhanced occurrence of breast and Leydig cell tumors together with an enhanced expression of ERα in the tumor tissue (6). The latter findings reasonably address how a short estrogen autocrine loop may be involved in breast and testicular tumorigenesis in the presence of an excess of locally produced estradiol. Indeed, an arrest of cell growth was observed following abrogation of local E_2 production with letrozole or after addition of ERα inhibitors ICI 182780 or 4-hydroxytamoxifen. Besides, only after changing the medium everyday along with prolonged R2C starvation, abolishing local steroid production, did we observe how exogenous E_2 was able to display proliferative effects.

One mechanism through which estrogens induce cell proliferation is by increasing protein levels of G_1 regulatory cyclins A, B1, D1, D3, and E in target cells (33). In our study, we showed that the expression of two of the most important regulators of Leydig cell cycle, cyclin D1 and cyclin E, can be increased by E_2 and down-regulated by treatment with antiestrogens. These data further confirm that aromatase overexpression and the consequent E_2 production may be the cause of altered cell cycle regulation of Leydig tumor cells.

In an attempt to explain the molecular mechanism determining aromatase overexpression in our tumor cell line, we focused our attention on the expression levels of transcription factors identified as crucial regulators of aromatase gene expression, CREB and SF-1. In the adult testis, SF-1 is predominantly expressed in Leydig cells (34). The increase of total and/or phosphorylated protein can potentiate SF-1 transcriptional activity (35). In R2C cells and in FRTT, compared with the normal controls, we found higher phosphorylated SF-1 protein levels as a consequence of elevated protein content. Total CREB levels were similar in all samples but highly phosphorylated in tumor samples. Starting from these observations, we investigated which pathways might be involved in the activation of these transcription factors.

The most important signal regulating Leydig cell function is the binding of LH to the LH receptor (LHR; ref. 36). Several observations indicate that constitutively active mutants of LHRs could be involved in Leydig cell transformation (37). It has been shown that the LH/LHR signaling pathway is constitutively active in the R2C tumor Leydig cell line and makes the phenotype of these cells constitutively steroidogenic (38). For instance, in the presence of a specific protein kinase A (PKA) inhibitor, constitutive syntheses of *Star* mRNA and steroids were significantly inhibited (39). These observations fit well with our findings indicating how the presence of PKA inhibitor determined a strong decrease in aromatase activity, together with a drop in CREB phosphorylation (data not shown). Here, we show that the presence of a specific PKC inhibitor had no effects on CREB phosphorylation while SF-1 dropped dramatically.

It has been shown that CREB in mouse Leydig cells can be phosphorylated also through the PKC pathway activated by IGF-I (38). In this work, we have revealed that R2C tumor Leydig cells release higher levels of IGF-I in the culture medium with respect to TM3 cells, and the concentration of IGF-I in FRTT is increased with respect to FRNT. However, the exposure to IGF-I as well as to the treatment with inhibitors of IGF-I signaling did not affect the CREB phosphorylative status but decreased SF-1 phosphorylation, postulating separate mechanisms that control CREB and SF-1 activation in modulating aromatase activity.

It has been suggested that IGF-I can influence Leydig cell survival and proliferation (40, 41). Moreover, it has been shown that IGF-I up-regulates aromatase expression in primary cultures of rat Leydig cells through a cAMP-independent mechanism (42). Our findings led us to suppose that the elevated IGF-I levels derived from tumor Leydig cells *in vivo* and *in vitro* contribute to enhance aromatase expression through an autocrine mechanism activating SF-1. The important role played by IGF-I in Leydig cell tumorigenesis is further supported by the substantially unchanged IGF-IR expression level between TM3 and R2C cells. Binding of IGF-I to its receptor causes receptor autophosphorylation and the activation of an intrinsic tyrosine kinase that acts on various substrates, leading to activation of multiple signaling pathways including the PI3K/AKT and MAPK cascades. In addition, it has been shown that IGF-I can activate the PLC/PKC pathway (18). We treated R2C cells with specific inhibitors of IGF-I signaling (AG1024), ERK1/2 (PD98059), PI3K (LY294002), and PKC (GF109203X) in the presence or absence of IGF-I and revealed that addition of IGF-I itself was able to increase aromatase expression, activity, and estradiol production, whereas all the inhibitors determined a reduction of enzyme activity and estradiol release.

For instance, the inhibition of IGF-I signaling through inhibition of either the PI3K/AKT or PLC/PKC pathway was able to block SF-1 expression and protein phosphorylation. In particular, treatment with AG1024 blocked SF-1 phosphorylation more efficiently than the separate treatment with PI3K or PKC alone, addressing how both pathways may synergize in up-regulating SF-1 activity. In the presence of PD98059, SF-1 expression remained unchanged as did aromatase mRNA and protein levels. Importantly, aromatase activity and estradiol production seemed to be decreased in the presence of PD98059, suggesting a potential stimulatory role of ERK1/2 on the enzyme at a posttranscriptional level. Furthermore, we observed that SF-1 binding to the aromatase promoter II is enhanced by IGF-I and reduced by AG1024, LY294002, and GF109203X, but not by PD98059, indicating the central role of this transcription factor in regulating aromatase gene transcription in tumor Leydig cells. This is the first report of a direct link between SF-1 transcription and the IGF-I signaling pathway in regulating aromatase expression.

The observed changes in estradiol production due to IGF-I determined an effect on R2C cell proliferative behavior. In fact, inhibitors of IGF-I signaling or the use of an anti–IGF-I antibody in immunoneutralization experiments blocked thymidine incorporation. Moreover, IGF-I up-regulates cyclins D1 and E whereas IGF-I signaling inhibitors decrease the same factors, analogously to the antiestrogen ICI 182780.

From our findings emerges a double mechanism inducing enhanced expression of aromatase: (*a*) constitutive activation of the LH/cAMP/PKA pathway, which determines CREB activation; (*b*) enhanced IGF-I signaling potentiating SF-1 action. The enhanced activity of SF-1 in inducing aromatase expression may be maintained by the lack of DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome, gene 1) expression in R2C cells (29). DAX-1 is a specific corepressor of SF-1 (43, 44) and inhibits StAR expression and steroidogenesis by 40% to 60% when overexpressed in R2C cells (29). The lack of DAX-1 expression in R2C cells may be due to constitutively active PKA signaling because in a mouse Leydig cell line, a marked decrease of DAX-1 mRNA occurred within 3 h after addition of LH and forskolin (45). Then, the activation of LH/LHR/PKA pathway at the same time decreases DAX-1 expression and promotes SF-1 activity. A further demonstration of the role of DAX-1 in regulating *P*450 aromatase expression comes from the observations that in TM3 cells, IGF-I induces SF-1 expression but is unable to induce aromatase expression because DAX-1 is highly expressed (data not shown). Finally, the finding that in DAX-1 knockout mice aromatase is overexpressed selectively in Leydig cells (46) underscores the importance of this type of transcription factor in local testicular estrogen production *in vivo*.

It remains to be explained which molecular mechanism(s) is responsible for the elevated IGF-I production in Leydig tumor cells. *In vivo*, the administration of human CG increases IGF-I mRNA levels in rat Leydig cells (47). LH deprival determines a decrease in bromodeoxyuridine incorporation as well as a decrease in IGF-I and IGF-IR mRNA levels (48). These observations suggest the possibility that LH can mediate its proliferative effects also by regulating IGF-I and its receptor in Leydig cells and that the altered LH/LHR–activated pathway in R2C cells could be the cause of IGF-I overproduction. Moreover, the observation that in murine Leydig cells IGF-I is able to increase LHR mRNA stability (49), together with data showing that the presence of an anti–IGF-I antibody reduces the steroidogenic responsiveness to LH/human CG (50), also suggests the possibility of IGF-I action in sustaining LH/LHR signaling. Aromatase overexpression seems to be induced by the combined enhancement of LH/LHR and IGF-I signaling. Particularly, LH/LHR signaling determines a constitutive active CREB phosphorylation on aromatase gene promoter whereas IGF-I overproduction stimulates SF-1 binding on the same promoter through an autocrine mechanism. In other words, from this study, it emerges that the reproducibility of data between our *in vivo* and *in vitro* models is linked to an enhanced PKA activity, together with increased E_2/ERα and IGF-I signaling.

In conclusion, in this study we showed that in Leydig tumor cells, aromatase overexpression determines an excessive local estradiol production that is able to stimulate the expression of genes involved in cell cycle regulation sustaining cell proliferation. Aromatase overexpression seems to be concomitant with an enhanced IGF-I signaling in R2C cells as well as in our *in vivo* model, supporting the hypothesis of a cooperation between estrogen and IGF-I in Leydig cell tumorigenesis, which is also observed in other tumor tissues. The observation that antiestrogens and aromatase inhibitors as well as IGF-I signaling blockers are able to reduce R2C proliferation is indicative of possible applications of these drugs as new adjuvant therapeutic tools for the treatment of testicular cancer.

Acknowledgments

Received 11/2/2006; revised 5/9/2007; accepted 6/20/2007.

Grant support: PRIN-MIUR grant no. 2004067227 and Associazione Italiana per la Ricerca sul Cancro (AIRC).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. V. Cunsulo (Biogemina Italia S.R.L., Catania-Italy) for generously providing us with the RIA kits, Dr. Ken-ichirou Morohashi and Dr. Holly A. Ingraham for providing us respectively with anti–SF-1 and anti–pSF-1 antibodies, and Dr. Soluzzo Cavalcanti for his technical and scientific contribution.

References

1. Carroll PR, Whitmore WF, Jr., Herr HW, et al. Endocrine and exocrine profiles of men with testicular tumors before orchiectomy. J Urol 1987;137:420–3.
2. Hawkins C, Miaskowski C. Testicular cancer: a review. Oncol Nurs Forum 1996;23:1203–11.
3. Mostofi FK, Sesterhenn IA, Bresler VM. Pathology of tumours in laboratory animals. Tumours of the rat. Tumours of the testis. IARC Sci Publ 1990;99:399–419.
4. Horn HA, Stewart HL. A review of some spontaneous tumors in noninbred mice. J Natl Cancer Inst 1952;13: 591–603.
5. Bosland MC. Hormonal factors in carcinogenesis of the prostate and testis in humans and in animal models. Prog Clin Biol Res 1996;394:309–52.
6. Fowler KA, Gill K, Kirma N, Dillehay DL, Tekmal RR. Overexpression of aromatase leads to development of testicular Leydig cell tumors: an *in vivo* model for hormone-mediated testicular cancer. Am J Pathol 2000; 156:347–53.
7. Kirma N, Gill K, Mandava U, Tekmal RR. Overexpression of aromatase leads to hyperplasia and changes in the expression of genes involved in apoptosis, cell cycle, growth, and tumor suppressor functions in the mammary glands of transgenic mice. Cancer Res 2001;61:1910–8.
8. Coleman GL, Barthold W, Osbaldiston GW, Foster SJ, Jonas AM. Pathological changes during aging in barrier-reared Fischer 344 male rats. J Gerontol 1977;32:258–78.
9. Aquila S, Sisci D, Gentile M, et al. Towards a physiological role for cytochrome *P*450 aromatase in ejaculated human sperm. Hum Reprod 2003;18:1650–9.
10. Inkster S, Yue W, Brodie A. Human testicular aromatase: immunocytochemical and biochemical studies. J Clin Endocrinol Metab 1995;80:1941–7.
11. Simpson ER, Mahendroo MS, Means GD, et al. Aromatase cytochrome *P*450, the enzyme responsible for estrogen biosynthesis. Endocr Rev 1994;15:342–55.
12. Young M, Lephart ED, McPhaul MJ. Expression of aromatase cytochrome *P*450 in rat H540 Leydig tumor cells. J Steroid Biochem Mol Biol 1997;63:37–44.
13. Lanzino M, Catalano S, Genissel C, et al. Aromatase messenger RNA is derived from the proximal promoter of the aromatase gene in Leydig, Sertoli, and germ cells of the rat testis. Biol Reprod 2001;64:1439–43.
14. Young M, McPhaul MJ. A Steroidogenic actor-1-binding site and cyclic adenosine 3′,5′-monophosphate response element-like elements are required for the activity of the rat aromatase promoter in rat Leydig tumor cell lines. Endocrinology 1998;139:5082–93.
15. Fitzpatrick SL, Richards JS. Identification of a cyclic adenosine 3′,5′-monophosphate-response element in the rat aromatase promoter that is required for transcriptional activation in rat granulosa cells and R2C Leydig cells. Mol Endocrinol 1994;8:1309–19.
16. Carlone DL, Richards JS. Functional interactions, phosphorylation, and levels of 3′,5′-cyclic adenosine monophosphate-regulatory element binding protein and Steroidogenic Factor-1 mediate hormone-regulated and constitutive expression of aromatase in gonadal cells. Mol Endocrinol 1997;11:292–304.
17. Fitzpatrick SL, Richards JS. *cis*-acting elements of the rat aromatase promoter required for cyclic adenosine 3′,5′-monophosphate induction in ovarian granulosa cells and constitutive expression in R2C Leydig cells. Mol Endocrinol 1993;7:341–54.
18. Manna PR, Chandrala SP, King SR, et al. Molecular mechanisms of insulin-like growth factor-I mediated regulation of the steroidogenic acute regulatory protein in mouse Leydig cells. Mol Endocrinol 2006;20:362–78.
19. Saez JM. Leydig cells: endocrine, paracrine, and autocrine regulation. Endocr Rev 1994;15:574–626.
20. Casella SJ, Smith EP, van Wyk JJ, et al. Isolation of rat testis cDNAs encoding an insulin-like growth factor I precursor. DNA 1987;6:325–30.
21. Zhou J, Bondy C. Anatomy of the insulin-like growth factor system in the human testis. Fertil Steril 1993;60: 897–904.
22. Baker J, Hardy MP, Zhou J, et al. Effects of an Igf1 gene null mutation on mouse reproduction. Mol Endocrinol 1996;10:903–18.
23. Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell 1993;75:59–72.
24. Wang GM, O'Shaughnessy PJ, Chubb C, Robaire B, Hardy MP. Effects of insulin-like growth factor i on steroidogenic enzyme expression levels in mouse Leydig cells. Endocrinology 2003;144:5058–64.

25. Lephart ED, Simpson ER. Assay of aromatase activity. Methods Enzymol 1991;206:477–83.

26. Wolanska M, Bankowski E. An accumulation of insulin-like growth factor I (IGF-I) in human myometrium and uterine leiomyomas in various stages of tumour growth. Eur Cytokine Netw 2004;15:359–63.

27. Pezzi V, Sirianni R, Chimento A, et al. Differential expression of steroidogenic factor-1/adrenal 4 binding protein and liver receptor homolog-1 (LRH-1)/fetoprotein transcription factor in the rat testis: LRH-1 as a potential regulator of testicular aromatase expression. Endocrinology 2004;145:2186–96.

28. Jacobs BB, Huseby RA. Neoplasms occurring in aged Fischer rats, with special reference to testicular, uterine and thyroid tumors. J Natl Cancer Inst 1967;39:303–9.

29. Jo Y, Stocco DM. Regulation of steroidogenesis and steroidogenic acute regulatory protein in R2C cells by DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome, gene-1). Endocrinology 2004;145:5629–37.

30. Pomerantz D. Developmental changes in the ability of follicle stimulating hormone to stimulate estrogen synthesis *in vivo* by the testis of the rat. Biol Reprod 1980;23:948–54.

31. Genissel C, Carreau S. Regulation of the aromatase gene expression in mature rat Leydig cells. Mol Cell Endocrinol 2001;178:141–6.

32. Valensi P, Coussieu C, Pauwles A, et al. Feminizing Leydig cell tumor: endocrine and incubation studies. J Endocrinol Invest 1987;10:187–93.

33. Prall OWJ, Sarcevic B, Musgrove EA, Watts CKW, Sutherland RL. Estrogen-induced activation of Cdk4 and Cdk2 during G_1-S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E-Cdk2. J Biol Chem 1997;272:10882–94.

34. Hatano O, Takayama K, Imai T, et al. Sex-dependent expression of a transcription factor, Ad4BP, regulating steroidogenic P-450 genes in the gonads during prenatal and postnatal rat development. Development 1994;120:2787–97.

35. Desclozeaux M, Krylova IN, Horn F, Fletterick RJ, Ingraham HA. Phosphorylation and intramolecular stabilization of the ligand binding domain in the nuclear receptor steroidogenic factor 1. Mol Cell Biol 2002;22:7193–203.

36. Dufau ML, Winters CA, Hattori M, et al. Hormonal regulation of androgen production by the Leydig cell. J Steroid Biochem 1984;20:161–73.

37. Ascoli M. Potential Leydig cell mitogenic signals generated by the wild-type and constitutively active mutants of the lutropin/choriogonadotropin receptor (LHR). Mol Cell Endocrinol 2007;260–262:244–8.

38. Jo Y, King SR, Khan SA, Stocco DM. Involvement of protein kinase C and cyclic adenosine 3′,5′-monophosphate-dependent kinase in steroidogenic acute regulatory protein expression and steroid biosynthesis in Leydig cells. Biol Reprod 2005;73:244–55.

39. Rao RM, Jo Y, Leers-Sucheta S, et al. Differential regulation of steroid hormone biosynthesis in R2C and MA-10 Leydig tumor cells: role of SR-B1-mediated selective cholesteryl ester transport. Biol Reprod 2003;68:114–21.

40. Colon E, Zaman F, Axelson M, et al. Insulin-like growth factor-I is an important antiapoptotic factor for rat Leydig cells during postnatal development. Endocrinology 2007;148:128–39.

41. Wang G, Hardy MP. Development of Leydig cells in the insulin-like growth factor-I (IGF-I) knockout mouse: effects of IGF-I replacement and gonadotropic stimulation. Biol Reprod 2004;70:632–9.

42. Rigaudiere N, Grizard G, Boucher D. Aromatase activity in purified Leydig cells from adult rat. Comparative effects of insulin, IGF-I, and hCG. Acta Endocrinol Copenh 1989;121:677–85.

43. Bae DS, Schaefer ML, Partan BW, Muglia L. Characterization of the mouse DAX-1 gene reveals evolutionary conservation of a unique amino-terminal motif and widespread expression in mouse tissue. Endocrinology 1996;137:3921–7.

44. Osman H, Murigande C, Nadakal A, Capponi AM. Repression of DAX-1 and induction of SF-1 expression. Two mechanisms contributing to the activation of aldosterone biosynthesis in adrenal glomerulosa cells. J Biol Chem 2002;277:41259–67.

45. Song KH, Park YY, Park KC, et al. The atypical orphan nuclear receptor DAX-1 interacts with orphan nuclear receptor Nur77 and represses its transactivation. Mol Endocrinol 2004;18:1929–40.

46. Wang ZJ, Jeffs B, Ito M, et al. Aromatase (Cyp19) expression is up-regulated by targeted disruption of Dax1. Proc Natl Acad Sci U S A 2001;98:7988–93.

47. Moore A, Chen CL, Davis JR, Morris ID. Insulin-like growth factor-I mRNA expression in the interstitial cells of the rat testis. J Mol Endocrinol 1993;11:319–24.

48. Sriraman V, Rao VS, Sairam MR, Rao AJ. Effect of deprival of LH on Leydig cell proliferation: involvement of PCNA, cyclin D3 and IGF-1. Mol Cell Endocrinol 2000;162:113–20.

49. Zhang FP, El-Hafnawy T, Huhtaniemi I. Regulation of luteinizing hormone receptor gene expression by insulin-like growth factor-I in an immortalized murine Leydig tumor cell line (BLT-1). Biol Reprod 1998;59:1116–23.

50. Le Roy C, Lejeune H, Chuzel F, Saez JM, Langlois D. Autocrine regulation of Leydig cell differentiated functions by insulin-like growth factor I and transforming growth factor β. J Steroid Biochem Mol Biol 1999;69:379–84.