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A cross-talk between estrogens and IGFIR pathways controls Leydig and adrenocortical tumor cell proliferation

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Index

Rationale I

Substance abuse has become increasingly widespread among athletes at sub competitive and recreational level, raising concern for human health. In addition to the illicit use of substances to increase performance of athletes and to enhance the muscular mass and strength, more recently the use of some agents has been extended to non-athletes with the aim to combat ageing, obesity and improve appearance or libido (1). Since its discovery in 1935, numerous derivatives of testosterone have been synthesized, with the goals of prolonging its biological activity in vivo, producing orally active androgens, and developing products, commonly referred to as anabolic androgenic steroids (AAS), that are more anabolic and less androgenic than the parent molecule. AAS doping is undeniably rampant worldwide. The doses of testosterone or other androgens used by athletes are substantially larger than those prescribed for the treatment of androgen deficiency. In one survey (2), 50% of androgen users reported using at least 500 mg of testosterone weekly or an equivalent dose of another androgen; in another survey (3), almost one fourth of androgen users assumed 1000 mg of testosterone weekly or an equivalent dose of other androgens. It is becoming increasingly clear that the abuse of AAS is associated with serious adverse effects to the liver (4) and the cardiovascular (5) central nervous (6), musculoskeletal (7), endocrine (6) and reproductive (8, 9) systems. As a consequence of their effects on the endocrine and reproductive systems AAS cause suppressed spermatogenesis, gynecomastia and virilization. Clinical reports highlight a link between AAS abuse and various types of cancer, mainly to the liver such as hepatocellular adenomas and adenocarcinomas (10), however other types of cancer such as Wilms' tumors have been reported (11, 12).

Androgens exert their biological effect through an intracellular receptor, the androgen receptor (AR), that is present in the reproductive tract as well as in many non-reproductive tissues, including bone, skeletal muscle, brain, liver, kidney and adipocytes. Binding of androgens to AR determines receptor dimerization, nuclear translocation and binding to specific responsive elements (ARE) present in the promoter region of target genes (13). Androgens mechanism of action in skeletal muscle cells is well documented and includes up-regulation of markers of myogenic

differentiation, such as MyoD and myosin heavy chain II (14-16). However, androgens can be converted to estrogens through the action of the aromatase enzyme. In the human, aromatase is expressed in a number of cells including brain, skin fibroblasts, bone, adipose tissue, in steroidogenic tissues such as placenta and gonads (17), in particular in man aromatase is present in most of the testicular cells.

Estrogens are required for a normal spermatogenesis, which seems extremely sensitive to estrogen concentration. Transgenic mice lacking aromatase expression (ArKO mice) show an age-dependent disruption of spermatogenesis, a significant reduction in testis weight and compromised fertility (18, 19). Similarly, men with inactivating mutations of the aromatase gene, leading to the lack of the estrogen synthesis, are infertile (20). On the other hand, about half of the male transgenic mice over-expressing aromatase and presenting enhancement of circulating 17β-estradiol (E2) levels are infertile and/or have enlarged testis and show Leydig cell hyperplasia and Leydig cell tumors (21). Several studies indicated that estrogen produced locally can induce neoplastic changes in breast tissue (22), and that their excess in rodents is able to stimulate Leydig cell hyperplasia (LCH) associated with cryptorchidism, testicular cancer and alterations of spermatogenesis (23-25). In a previous study it has been shown that Leydig cell tumor is characterized by aromatase overexpression and consequent increased estrogen production, that contributes to inducing tumor cell proliferation (26). Aromatase activity is regulated mainly at the level of gene expression and is present throughout all maturational stages of the male gamete in humans (27, 28).

Recently it was shown that SF-1 (steroidogenic factor-1) is localized predominantly in Sertoli cells, LRH-1 (liver receptor homologue -1) is present in germ cells, while both transcription factors are present in the cells of primary rat Leydig and positively regulate the expression of the aromatase (29). Increased expression of transcription factors acting on the aromatase promoter PII, including SF-1, is at the basis of the mechanism of proliferation induced by IGF-1 on Leydig tumor cells (30) because it establishes an autocrine mechanism by which IGF-1 up-regulates aromatase through SF-1 activation. The consequent synthesis of estrogens is responsible for the induction of cyclins and cell proliferation.

Testosterone, nandrolone, stanozolol, methandienone, and methenolol are the most frequently abused androgens (2, 3, 31). These androgens can be differentially metabolized by aromatase, specifically nandrolone can be converted to estrogens, while stanozolol is a non-aromatizable androgen.

In addition to the use of androgens, athletes also abuse other drugs to purportedly enhance muscle building, muscle shaping or athletic performance (2). These accessory drugs include stimulants, such as amphetamine, clenbuterol, ephedrine, and thyroxine, anabolic agents such as growth hormone (GH), insulin and insulin-like growth factor-I (IGF-I) and drugs perceived to reduce adverse effects such as human chorionic gonadotropin (hCG), aromatase inhibitors or estrogen antagonists (2). In particular, IGF-I, which is the main effector for the action of GH, is a peptide physiologically produced by the liver. The potential benefits of IGF-I administration include increased muscle protein synthesis and the sparing of glycogenolysis with glycogen synthesis and increased fatty acid availability. IGF-I is known to have a role in testicular growth and development and in the control of Leydig cell number (32). IGF-I is produced locally in the testis, in Sertoli, Leydig and peritubular cells derived from the immature testis and cultured in vitro (33). The crucial role of IGF-I in the development and function of Leydig cells was highlighted by studies on IGF-I gene knockout mice (34, 35). The failure of adult Leydig cells to mature and the reduced capacity for testosterone production in IGF-I knock out (KO) mice are caused by deregulated expression of testosterone biosynthetic and metabolizing enzymes (36), expression levels of all mRNA species associated with testosterone biosynthesis are lower in the absence of IGF-I. Furthermore, IGF-I plays a central role in inducing aromatase expression in Leydig tumor cells, consequently IGF-I increases estrogen production that contributes to the induction of tumor cell proliferation (26).

Starting from these observations and taking into account that Leydig cell tumors are common in young men, the same age group commonly abusing AAS we wanted to investigate the effects of AAS and IGF-I on Leydig cell tumors. Our hypothesis is that AAS can induce Leydig cell tumor proliferation and that this effect could be potentiated by the concomitant use of IGF-I. To verify this hypothesis in the present study we evaluated on Leydig R2C cells the effects of commonly used ASS,

differentially metabolized by aromatase, such as nandrolone (aromatizable) and stanozolol (non-aromatizable), used alone or in association with IGF-I, on aromatase expression and Leydig cell tumor proliferation.

Background I

1. Endocrinology of male reproductive system

1.1 The testis: general structure

The human male reproductive system includes hypothalamic-pituitary-gonadals axis, epididymis, vas deferens, seminal vesicles, prostate and urethra. The testis is primarily composed by seminiferous tubules closely packed together and interstitial cells (37). The seminiferous tubules are composed by Sertoli cells that support germ cells during their maturation into spermatozoa and that create a blood-testis barrier, separate the germinal epithelium into basal and adluminal compartments. Sertoli cells are responsible for germ cells physical support and in addition they provide 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. Testes produce a large amount of spermatozoa through a complex process, known as spermatogenesis. 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 (38, 39). Immune cells, known to secrete a number of growth factors and cytokines, are part of the intratesticular communication pathways (40).

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 sex hormones.

These two functions occur into separate compartments within the testis:

1. the seminiferous tubules produce sperm;
2. the interstitial cells (i.e., Leydig cells) synthesize androgens (Fig. 1.1).

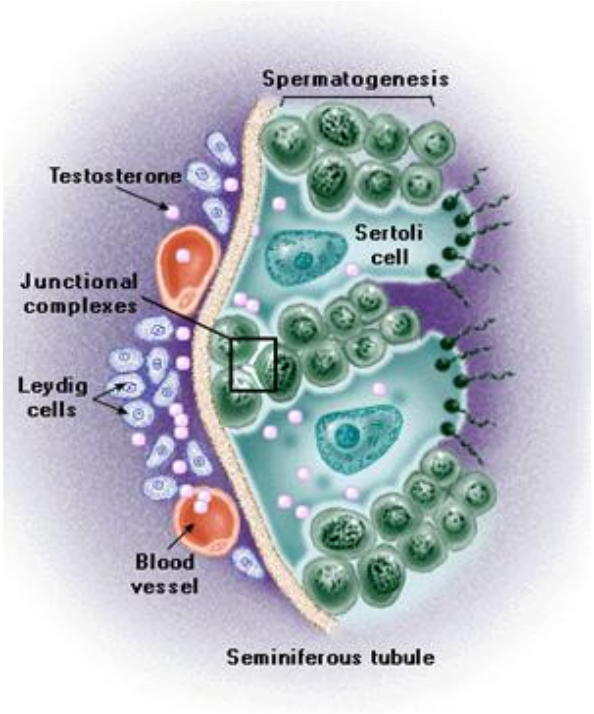


Figure 1.1. Schematic representation of functions of the testis.

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

1.3 The male hypothalamo-pituitary-gonadal axis

Secretion of gonadotrophins from the pituitary gland is responsible for regulating hormonal control of the gonad in the male (Fig. 1.2). The male hypothalamo-

pituitary-gonadal (HPG) axis is active from fetal life and the level of hormones produced varies at different stages throughout life. The axis regulates the onest of puberty and the establishment of spermatogenesis (41), in addition to the production of gonadal androgens. Gonadotrophin releasing hormone (GnRH) is produced by the hypothalamus and stimulates the secretion of two gonadotrophins from the anterior pituitary. These glycoprotein hormones are LH and follicle stimulating hormone (FSH). LH binds to the LH/CG receptor on the Leydig cells of the testis to promote testosterone secretion from the Leydig cells, and FSH acts on the Sertoli cells. Testosterone secreted by Leydig cells diffuses into the seminiferous tubules and in them, only Sertoli cells possess receptors for testosterone and FSH and so these cells are the major targets of the ultimate hormonal signals that regulate spermatogenesis.

Two important negative feedback loops exist to regulate the secretion of gonadotrophins. The testosterone negative feedback loop is established in fetal life and inhibits hypothalamic and pituitary production of GnRH and LH respectively (42). Negative feedback sensitivity of the HPG axis does not develop in the rat until late in gestation in (43) as LH is not present until 16.5 dpc in the rat (44), but after birth testosterone secretion is LH dependent in rat, human and marmoset (43). The other negative feedback loop results from production of Inhibin-B by the Sertoli cell, which exerts inhibitory effects on FSH secretion from the pituitary gland, however this negative feedback loop is only established at around puberty (45).

The profile of gonadotrophins and testosterone varies depending on age and development (Fig. 1.3). In the human during fetal life, the levels of testosterone are high with a peak at 14-17 weeks gestation (46). Following birth in humans and non-human primates there is an initial rise in gonadotrophins and testosterone that continues during early infancy, the so-called ‘mini puberty’ (47). In humans the rise begins at 2 weeks of life and peaks between 1 and 3 months of age (48), falling to low levels at 6-8 months. This pattern of secretion has also been demonstrated in many other primates, including the rhesus monkey and the marmoset (49, 50).

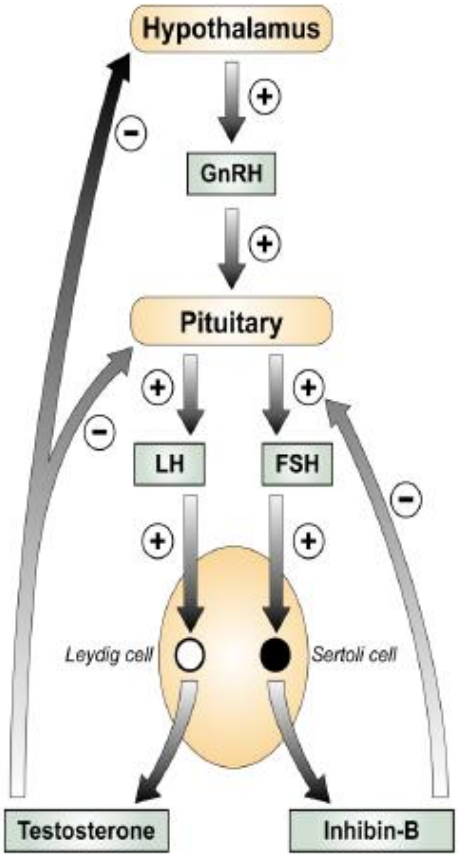


Figure 1.2. The male HPG axis. Gonadotrophins (LH and FSH) released from the anterior pituitary under the control of GnRH act on the testis to produce testosterone from the Leydig cell and Inhibin B from the Sertoli cell. Testosterone and Inhibin-B negatively feedback to the hypothalamus and/or pituitary. Stimulation (+) and inhibition (–) are indicated. Taken from (Mitchell et al., 2009).

In humans and non-human primates after the rise in gonadotrophins and testosterone during early infancy, there follows a period of relative 'quiescence' during which levels of these hormones are relatively low (50). This period will be referred to as the 'childhood period', which lasts from the end of infancy until the onset of puberty. Although this period has been described as a quiescent period it is clear that the testis is active. Sertoli cells actively express the FSH receptor, AMH and aromatase, which is the product of the CYP19 gene (51). In addition there are periods of germ cell proliferation (52) and the transient appearance of meiotic cells (51). Levels of the gonadotrophins and testosterone rise again peripubertally and remain high during adult life (Fig. 1.3). In rodents there is no equivalent childhood period of low gonadotrophin and testosterone (53).

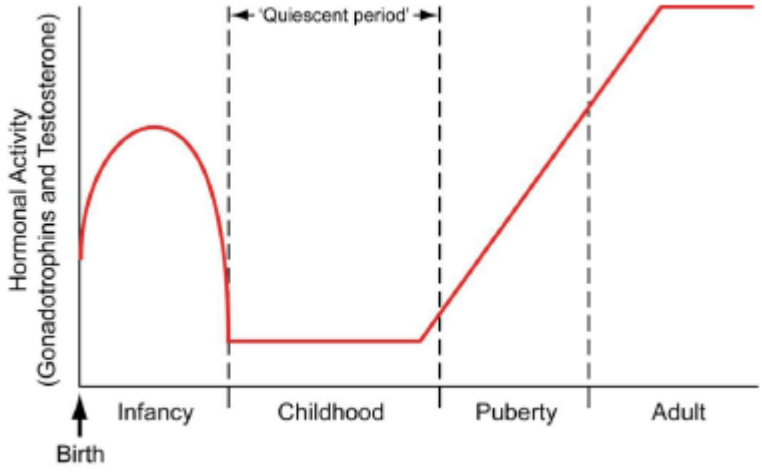


Figure 1.3. The profile of Gonadotrophin and testosterone secretion in primates. Taken from (Mitchell et al., 2009).

1.4 Endocrine regulation of the testis

The interaction of LH with its receptor initiates signalling through GTP binding proteins determining cyclic AMP (adenosine-3',5'-cyclic monophosphate) production (54) and signal transduction through protein kinase A pathway. Some data suggests that intracellular calcium concentration can be induced by the action of LH though the activation of the lipoxygenase pathway (55). In addition, changes in calcium concentration can also regulate adenylate cyclase through the protein kinase C pathway. FSH binding to its receptor is known to activate at least 5 signaling pathways in Sertoli cells: cAMP-PKA pathway, MAPK pathway, Phosphatidylinositol 3-kinase (PI3K) pathway, Calcium pathway, Phospholipase A2 (PLA2) pathway. Initially FSH binding to FSH receptor causes adenylate cyclase (AC) activation and increase in 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 MAPK cascade in Sertoli cells. ERK can activate transcription factors including SRF, c-jun and CREB. In granulosa cells, FSH also activates the p38 MAP kinase. FSH and cAMP also act through GEFs (guanine nucleotide exchange factors) to activate PI3K and then phosphoinositide dependant protein kinase (PDK1) and PKB in Sertoli cells.

Studies on granulosa cells identified Forkhead transcription factor (Forkhead), SGK (glucocorticoid-induced kinase) and GSK-3 (glycogen synthase kinase-3) as additional downstream targets of PI3K pathway.

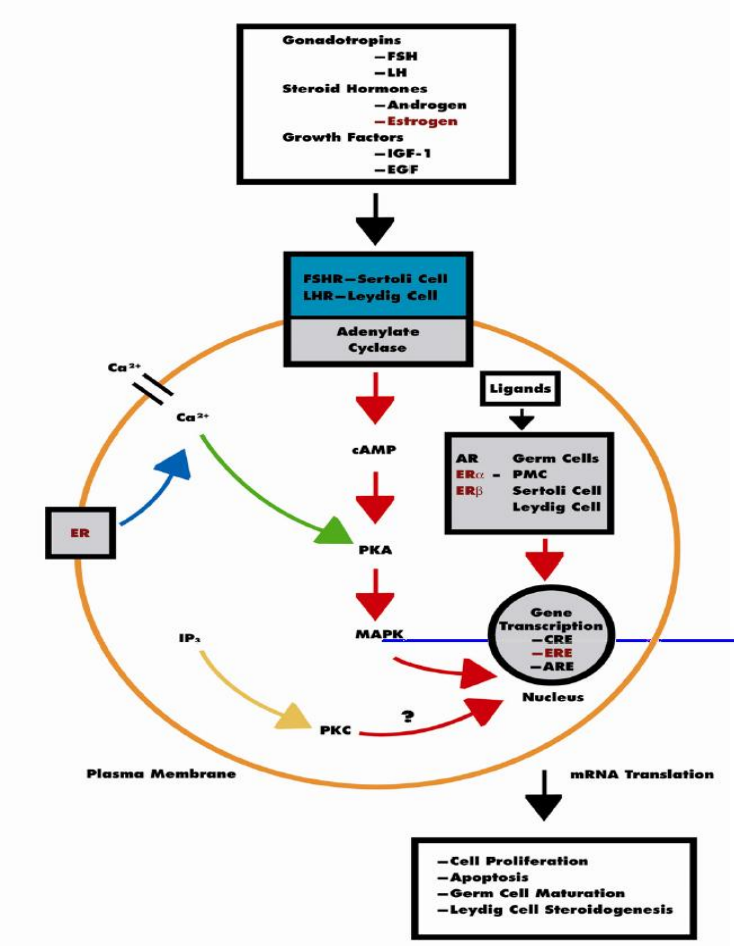


Figure 1.4. Endocrine regulation of the testis. PMC, peritubular myoid cell; CRE, cAMP responsive elements, ARE, androgen-responsive elements; ERE, estrogen-responsive elements**.**

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 (56). 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 (57). The pathway mediated by cAMP seems 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 their receptors, IGF-1R and EGF-R, can modulate AR and ER-mediated pathways. In conclusion is possible to affirm that testicular functions are regulated by interactions between several signaling pathways, some directly, 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 (Fig 1.4).

1.5 Spermatogenesis

Spermatogenesis is the process by which spermatogonia proliferate and then differentiate into mature spermatozoa. This process is initiated by FSH during puberty and both FSH and testosterone appear to be required for quantitatively normal spermatogenesis (41, 58, 59). Testosterone acts via the androgen receptor on the Sertoli cells, thereby exerting indirect effects on the germ cells, as evidenced by a reduced germ cell number and failure to progress beyond meiosis in mice with knockout of the androgen receptor in Sertoli cells (60). There are three phases of spermatogenesis that are common to all mammals. The first is a phase in which the spermatogonia are undergoing frequent cell divisions and differentiating into primary spermatocytes. The final mitotic division of B-spermatogonia gives rise to diploid preleptotene spermatocytes, which undergo meiotic division to produce haploid secondary spermatocytes. These cells are located in the adluminal compartment of the seminiferous tubule. The second meiotic division results in the formation of the spermatids, which will subsequently become mature spermatozoa following the process of spermiogenesis. The seminiferous tubule is organised with the spermatogonia adjacent to the basement membrane. As the germ cells differentiate they are directed towards the lumen (Fig. 1.5).

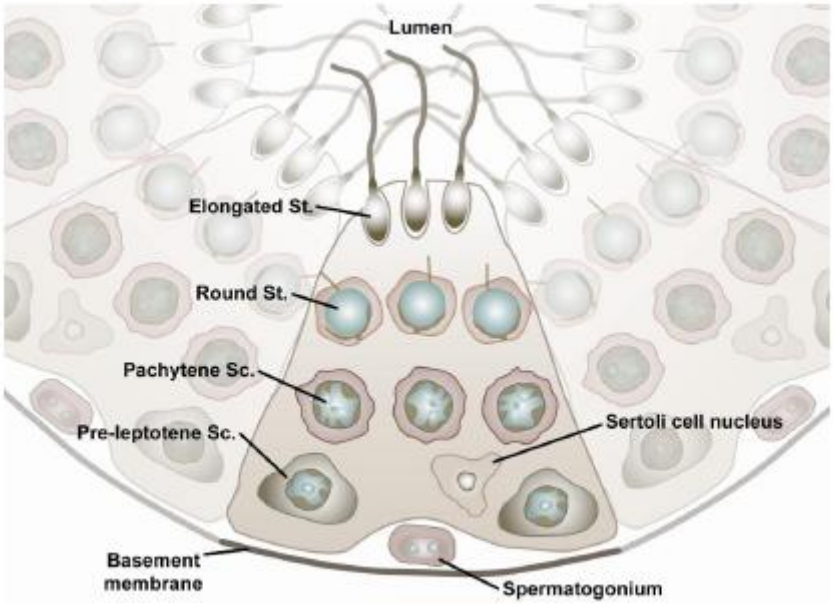


Figure 1.5. Schematic representation of a transverse view of a human seminiferous tubule. Sc – spermatocyte, St – spermatid. Supporting the germ cells are the Sertoli cells, which form the 'blood testis barrier' consisting of tight (occluding) junctions between adjacent Sertoli cells. Each Sertoli cell provides support for numerous germ cells at different stages of development and the function of the Sertoli cells at a given stage is determined by its germ cell complement. The patterns of germ cell association from basement membrane to the tubule lumen are classified into stages, which are based on the morphological development of the spermatids. Six stages have been described in the human.

In human adults the proliferation rate of the spermatogonia has been calculated as 26%, based on Ki67 staining. This is higher in type B spermatogonia (43%) compared to the type A spermatogonia (22%) (61). Each pachytene spermatocyte gives rise to four haploid spermatids and no further mitotic division occurs once the germ cells have reached this stage (41).

Germ cell numbers can be increased by administration of FSH in adult rhesus monkeys (62) and this has been shown to be due to proliferation of type Apale spermatogonia (59, 62). In these studies administration of hCG (62) or LH (59) did not increase cell proliferation, suggesting that the threshold for a maximal impact of androgens has already been met, whilst FSH administration can augment cell function. However the importance of testosterone can be inferred from studies in the *hpg* mouse which lacks gonadotrophins and therefore has an immature testicular phenotype without full spermatogenesis, but in which replacement of testosterone induces complete spermatogenesis despite undetectable FSH levels (63). In men with low FSH levels, hCG can re-initiate spermatogenesis, although the sperm

concentrations do not return to normal (64). This suggests that both FSH and LH are required for normal spermatogenesis although it would appear that FSH is not essential to the process, in fact men with an inactivating mutation of the FSH receptor have varying degrees of spermatogenic impairment but are not infertile (65).

1.6 Steroid production

Testosterone is the major androgen secreted by the testis In addition to testosterone, in smaller amounts, is produced dihydrotestosterone through the actions of the enzyme 5α-reductase. Testis also contributes approximately for 25% of 17β-estradiol total daily production through the local action of the enzyme aromatase which converts androgenic substrates in estrogens (66). Cholesterol represents the major substrate for androgen production in 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 (67). In addition, Leydig cells can 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, cholesterol is converted in pregnenolone through cholesterol side-chain cleavage enzyme (cytP450$_{SCC}$) actions (68) (Fig. 1.6).

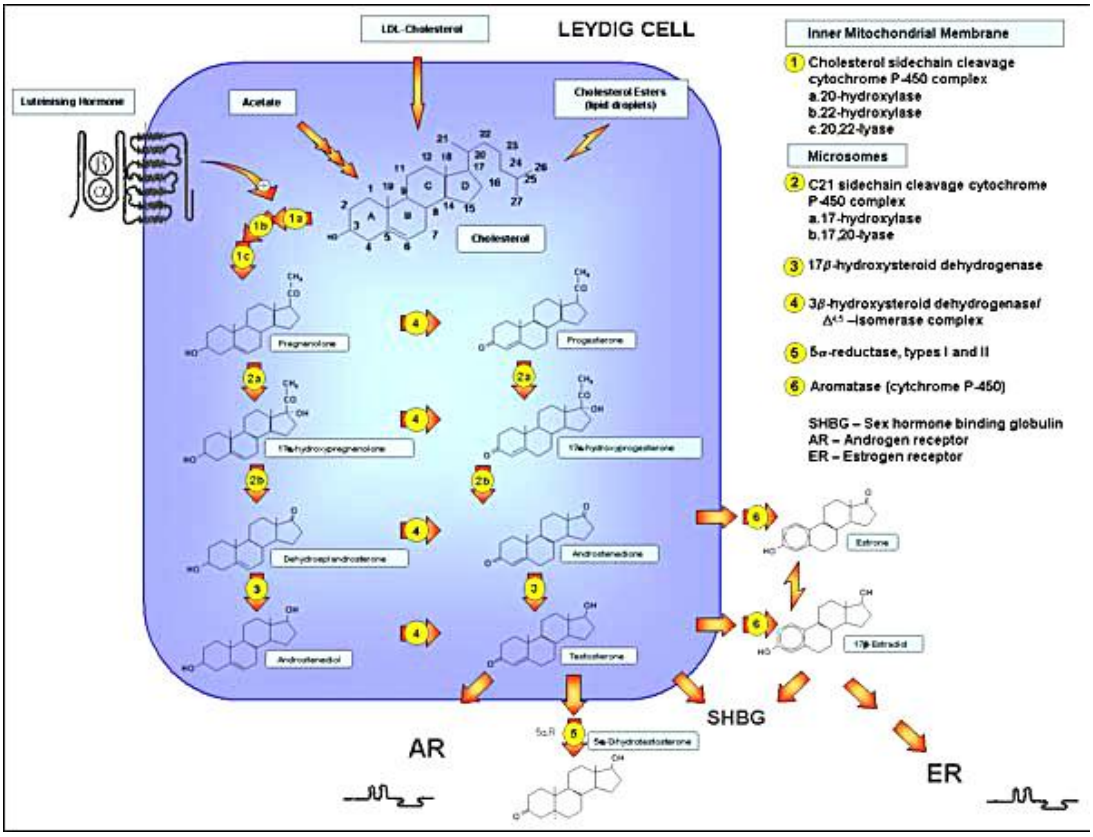


Figure 1.6. Steps in steroidogenesis leading to androgens and estrogens production.

Cholesterol conversion in pregnenolone, being the key step of androgen production, is tightly controlled Availability of cholesterol can be rate-limiting and the intracellular trafficking of cholesterol, across mitochondrial membranes, is dependent on the steroidogenic acute regulatory protein (STAR) (69-71).

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 are unable to convert cholesterol to pregnenolone (72). Furthermore, the results of studies involving targeted disruption of the mouse gene encoding STAR, support the data observed in human (73). Testosterone production can be obtainded through two pathways. Pregnenolone can be converted in progesterone through 3βhydroxysteroid dehydrogenase enzyme activity (Δ4 pathway) or can be hydroxylated by 17-αhydroxylase enzyme to form 17α-hydroxypregnenolone (Δ5 pathway). The relative importance of these two pathways vary with the species and the physiological status of the male (74). The further conversion of 17α-hydroxypregnenolone through the

Δ5 pathway involves the formation of a 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 (75, 76). The conversion of dehydroepiandrosterone in androstenediol is mediated by a microsomal enzyme, 17β-hydroxysteroid dehydrogenase (77, 78). Substrate conversions from the Δ5 to the Δ4 pathway are catalyzed by the enzyme 3β-hydroxysteroid dehydrogenase (79). In the Δ4 pathway, 17α-hydroxyprogesterone is converted in androstenedione and testosterone through the action of cytochrome $P450_{C17}$. Starting from testosterone the enzyme 5α-reductase can produce dihydrotestosterone (80) while the enzyme aromatase con produce 17β-estradiol (66, 81).

2.Testicular cancers

2.1 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 (82-85).

Because the incidence of testicular cancer has risen markedly in the past 20 years, researchers focused their attention on possible environmental causes, including mother's diet during the pregnancy as well as the increasing presence of estrogen-mimicking pollutants in the environment.

The most consistent occupational association has been the elevated rate among men in professional 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. Testicular germ cell tumours (TGCT) are the most common cancer of young men and represent 90% of testicular tumours (86). These tumours have increased in incidence during the last 50 years in many Western countries (87). TGCT have been postulated to arise during fetal life in humans (88), originating from the transformation of undifferentiated fetal germ cells (89). The exact origin and subsequent development of these pre-neoplastic germ cells is unknown. In addition there is a lack of suitable animal models of fetal and early postnatal testis development in which to investigate the origins of these tumours.

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, deriving from endocrine active cells, they have endocrine manifestations.

2.2 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 (90). 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 further progress to form Leydig cell tumors. The mechanism determining Leydig cell hyperplasia in 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 (90). In a small subset of cases structural changes of the LH receptor (91, 92) and G proteins (93, 94) were detected. Constitutively activating mutations of LH receptor cause early Leydig cell hyperplasia and precocious puberty (91, 95). Similarly, constitutively activating mutations of Gs-protein in Leydig cells lead into hyperplasia and endocrine hyperactivity (94, 96). 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 (92, 94). Leydig cell hyperplasia and adenomas can be easily induced in rodents by administration of estrogens, gonadotropins and a wide range of chemical compounds.

Leydig cell tumors account for one to three percent of testicular neoplasms and occur in different age groups (96-98). Approximately the 20% is 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 aromatase iper-activity and consequent excessive estrogen production. In adults, gynecomastia is found in approximately 30% of patients (98). 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 (84), whereas in adults malignant tumors have been found in 10-15% of patients, and inguinal orchidectomy remains the treatment of choice (97). 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 (98, 99). 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 (98, 99). Malignant tumors have not responded favorably to conventional chemotherapy and irradiation (98, 99). 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 (98, 99). 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(98-100).

The most frequently encountered testicular neoplasm in 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% (101). Early neoplasm are common in 1 yr old F-344 rats and become increasingly more frequent with age (102). Testicular neoplasia is less frequently observed in all 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 at any age, the majority between 20 and 60 years (103). 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 Leydig cell adenoma. The major difference between 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.

2.3 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 (21). 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 (104). This finding agrees with a single previous report (105) showing the aromatase immunolocalization in Leydig cell tumors. Furthermore, aromatase expression in control human testicular tissue confirmed Turner's report in normal testes (106). 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 two fold higher than those of adult normal male, and by the low testosterone levels (at the lower limit of normal range) (104). 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 (107) 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 circulating levels ratio, drops dramatically following surgical treatment, while for one of the two patients the persistence of a conspicuous bilateral gynecomastia led to bilateral mastectomia (104). Following orchidectomy, the two patients exhibited a moderate increase of sperm count and a remarkable augment of sperm motility (104). 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 (77); 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 (108, 109) .

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 (81). 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 (110). 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.

3. Estrogen regulation of testicular functions

3.1 Introduction

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:

1. aromatase localization and target protein for estrogen receptors (ERα and ERβ) in tissues of the reproductive tract;
2. analysis of testicular phenotypes in transgenic mice deficient in aromatase, ERα/ERβ gene;
3. investigation of environmental chemicals effects 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 (110). In males, estrogens derive from circulating androgens. Aromatization of 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. 3.1).

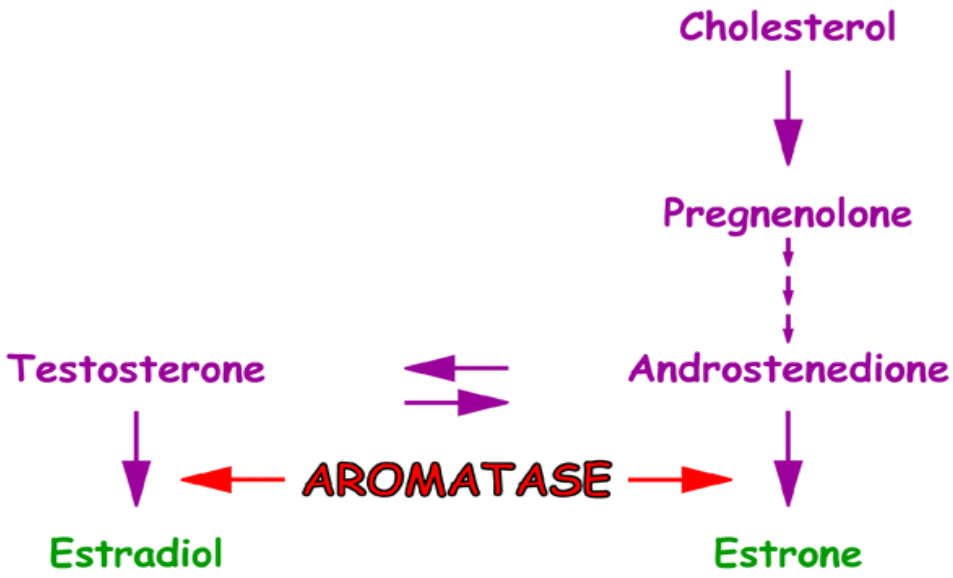


Figure 3.1. Biochemical pathway of testosterone conversion into estrogens.

3.2 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, $P450_{AROM}$ is the product of a single gene located in region q21.1 of the chromosome 15 and called *CYP19*. The *CYP19* gene is more than 123 kb in length with a coding region of 9 exons (II-X) and 9 nontranslated exons I (111) (Fig. 3.2).

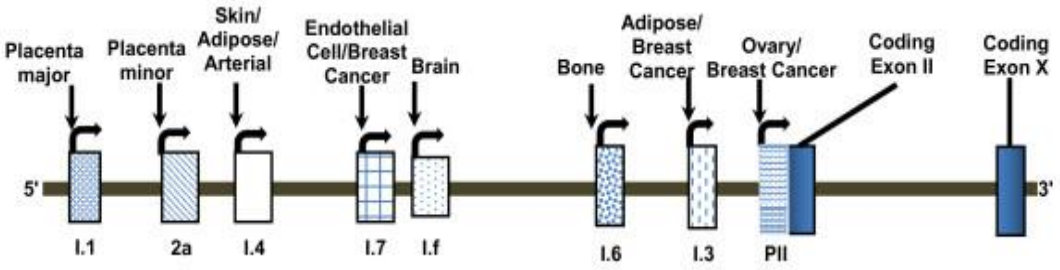


Figure 3.2. Schematic presentation of the human aromatase gene. P = promoter.

Expression of the *CYP19* gene is regulated by tissue-specific promoters producing alternate 5'-untranslated exons that are then spliced onto a common 3'- splice acceptor site in exon II, upstream of the translation starting site (17, 112, 113). 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 $P450_{AROM}$ is encoded by a single *CYP19* gene in most species except for pigs in which three distinct genes encode three aromatase isoenzymes (114) and for fish in which two genes (specifically expressed in the brain and gonads) have been identified (115). 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 function of different promoter elements (116). In human adipose tissue, the primary promoter I.4 lies about 15 kb upstream of the start site of translation (117, 118) and is a TATA-less promoter driven by glucocorticoids and class I cytokines e.g. IL-6 and TNFα (113). The region of PII proximal to the translation start site regulates $P450_{AROM}$ expression in mammalian gonads (117, 119)

as well as in Leydig cell tumors (120). Numerous functional motifs have been identified in P.II (113) (Fig. 3.3).

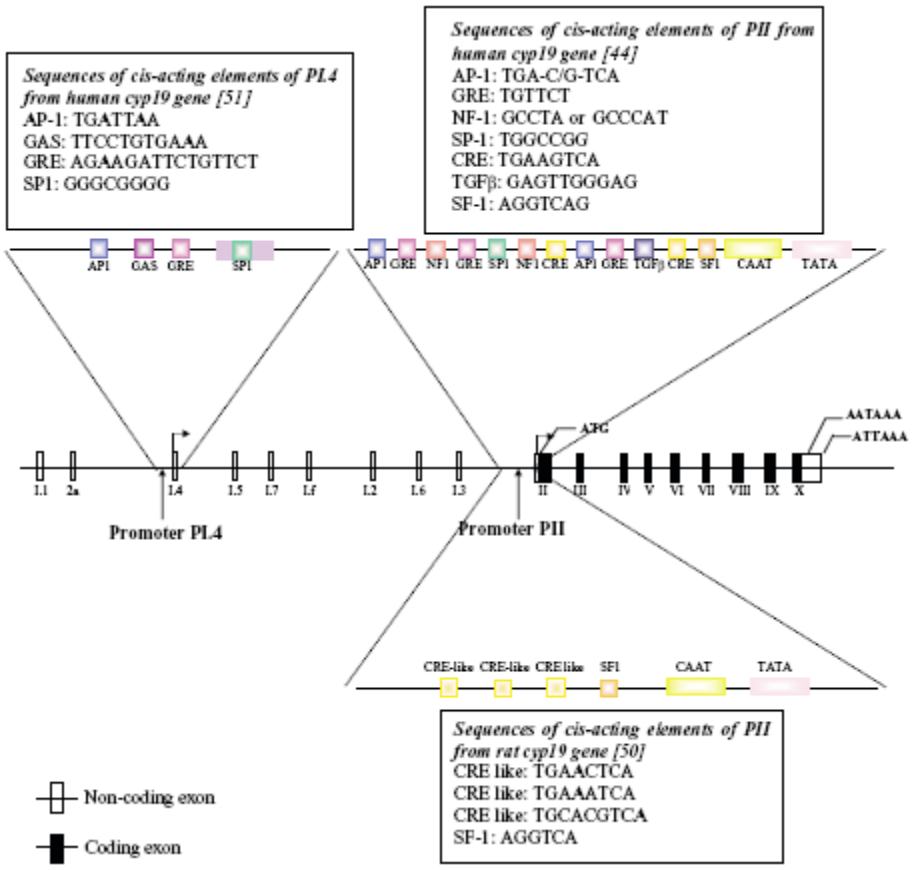


Figure 3.3. **Structure of the human Cyp19 gene showing the various untranslated first exons and their corresponding promoters.** The region around promoter PI.4 and PII from human and PII from rat are expanded to show the identified response elements. Sequences of these are shown in boxes.

In the testis, FSH and LH act by increasing concentrations of intracellular cyclic AMP to induce expression of $P450_{AROM}$. 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. $P450_{AROM}$ via its interaction with numerous coactivators including CREB binding protein, DAX-1, SOX-9, WT1).

It has been shown that $P450_{AROM}$ mRNA level is increased in Leydig cells of mice deficient for DAX-1 (121). In addition, it has been shown that liver receptor

homologue-1 (LRH-1), an SF-1 homologue, present in leydig and germ cells but not in sertoli cells, increases $P450_{AROM}$ gene expression in a mouse leydig cell line (29). Moreover, it is now clear that not only P.II drives aromatase gene in rat testis but that two additional promoters, P.I.f (brain promoter) and a new one that we called P.I.Tr (testis rat) (122), are involved. It is also demonstrated that the nutritional status of fetuses (123) and aging (124) can modulate aromatase gene expression in male rats.

3.2.1 The transcriptional factors CREB and SF1

CREB is a transcriptional factor binding the cAMP responsive elements (CRE). From a structural point of view it is possible to identify two regions:

the N-terminal, setting the process of transactivation;

the C-terminal, involved in DNA binding and in the process of dimerization.

The human protein consists of 327 amino acids (CREB-327) while in rats it consists of 341 amino acids (CREB-341); both are expressed by homologous genes and are the product of exons subject to alternative splicing.

The phosphorylation site(P-box) or kinase-inducible domain (KID) is the critical region of CREB in response to the cAMP. The P-box contains several consensus phosphorylation sites for different kinases such as PKA, PKC, glycogen synthase kinase 3 and the casein kinase I and II (CK).

After activation of adenylate cyclase pathway, PKA phosphorylates CREB at the level of serine in position 133.

Phosphorylation induces a conformational change in the protein (125). In addition to PKA, other signal transduction pathways target the CREB protein, increasing or decreasing its transcriptional activity. An example is the kinase IV Ca^{2+}-calmodulin-dependent (CaMKIV), which phosphorylates CREB at serine 133 after membrane depolarization in neuronal cells.

CREB phosphorylation can also be induced by signal transduction pathways triggered by growth factors and inflammatory cytokines.

The kinase II Ca^{2+}-calmodulin-dependent (CaMKII) phosphorylates CREB at serine 133 and serine 142, with a significant inhibition of CREB transactivation. In addition to calcium and cAMP, the phosphorylation of serine 133 also occurs through a transcriptional pathway Ras-dependent. PKC is able to phosphorylate CREB in vitro, but its role in vivo is doubtful; CREB phosphorylation in response to agents that

activate PKC may occur through the activation of mitogen-activated protein kinase (MAPK/RSK). SF-1 gene (steroidogenic factor 1) encodes for an orphan nuclear receptor that plays regulatory role in the endocrine function of the hypothalamic-pituitary-gonads, in adrenal gland and is an essential factor in sexual differentiation (126). SF-1 is present in the adrenal, Leydig, Sertoli and granulosa cells, in ovarian theca, in spleen, in anterior pituitary gland and in hypothalamus. It's well known that SF-1 plays an essential role in gonadal differentiation, because its expression in primordial gonads assumes different characteristics in male and female, in correspondence with the divergence of the development of the testis from that of the ovary. In mice lacking SF-1 gene (SF-1 KO), were found: agenesis of the gonads (127) and of the adrenal gland, a sexual change from male to female and external and internal genitalis, a damaged function of the gonadotropins and ablation of specific regions of the hypothalamus. Some studies suggest that SF-1 may also play a role in spermatogenesis as well as in steroidogenesis.The SF-1 DBD has two zinc-fingers and both, including also the P-box (Proximal box) in the first zinc-finger and the D-box (distal box) in the second zinc-finger, are highly conserved.

3.3 The Estrogen Receptors

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. 3.4).

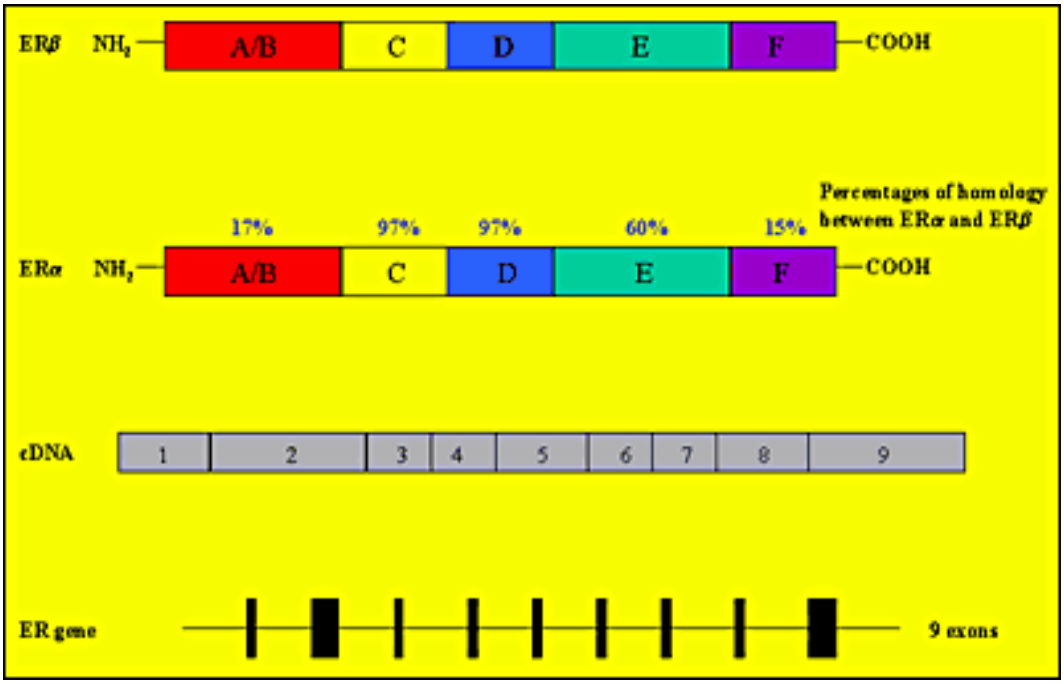


Figure 3.4. ERs gene and its products.

While it is clear that estrogens regulate transcription via a nuclear interaction after binding to 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 (128). 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. 3.4). 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 (129). 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 (130). 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 (131). 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 DNA binding domains of ERα and ERβ (about 95%), only a partial homology exists in the ligand-binding domain (~60%) (132). 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 (133-135). 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 (136). 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-I and EGF (137). 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* (138).

Recently, a large body of evidences has demonstrated that estrogens can function not only through ERs but can also trigger rapid responses that involve transduction pathways different from those activated “classically” by ERs (139-141).

Estrogen rapid signaling involves a series of cell typedependent events that include mobilization of second messengers such as calcium, cAMP, nitric oxide, interaction

with membrane receptors such as insulin-like growth factor 1 receptor (IGF1R) and epidermal growth factor receptor (EGFR), and stimulation of effector molecules, such as the Src family of tyrosine kinases, the PI3K, the serine/threonine protein kinase AKT and the mitogen-activated protein kinases (MAPKs) (142-146). Several studies suggest that rapid responses to estrogens can be mediated by classical receptors localized to the plasma membrane (139, 147, 148), or by transmembrane proteins other than ERs. Recently, our and other studies identified a transmembrane estrogen-binding protein, the G-protein coupled estrogen receptor 1 (GPR30 from GPER gene), which is able to mediate estrogen action (149-152).

GPR30 has been identified in a variety of human and rodent estrogen target tissues (153-156). Immunocytochemical studies have identified the intracellular localization of GPR30 in the endoplasmic reticulum, Golgi apparatus (151) and plasma membrane (157). GPR30 activates MAPKs, via transactivation of EGFR through release of heparin-bound epidermal growth factor (HB-EGF) (158). It also facilitates stimulation of adenylyl cyclase and cAMP-mediated attenuation of the EGFR-to-MAPK signaling axis (149). Using a *Gper*-lacZ reporter mouse Isensee *et al*. (159) demonstrated extensive expression of GPR30 in several endocrine organs including the testis. This is in agreement with our studies that revealed GPR30 expression in a spermatogonia mouse cell line (GC-1 cells) (30), in adult rat pachytene spermatocytes (PS) (160) and in rat round spermatids (RS) (161) and suggests a role for this receptor in controlling spermatogenesis.

Moreover, it has also been recently demonstrated GPR30 expression in Sertoli cells (162).

3.4 Distribution of ERs and aromatase in 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 (128). 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 (163).

3.4.1 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. Studies on the precise cellular localization of ERs, 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, 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. 3.5).

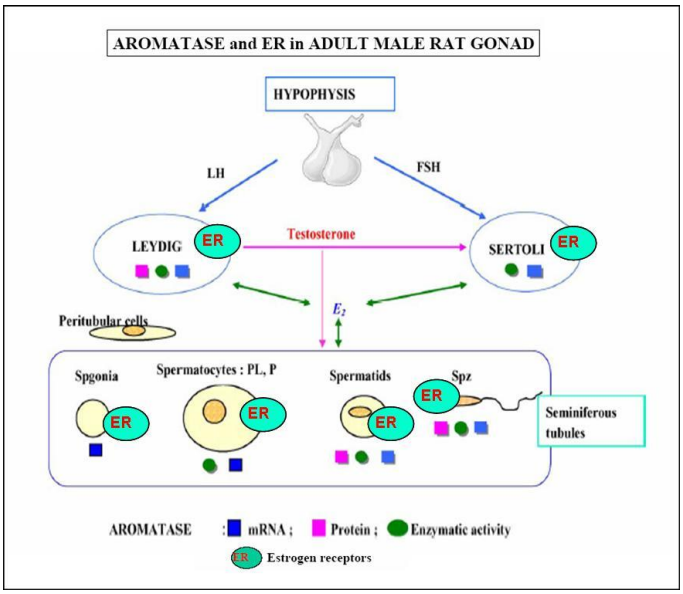


Figure 3.5. Aromatase and estrogen receptors (ER) in adult male rat gonad.

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.

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 persists 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).

3.4.2 ERs and aromatase in the human testis

Both ERs have been found in human testis and reproductive tract. In male fetus ERβ expression is higher than ERα, that is absent or expressed at very low levels. In 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 human epididymis is still debated, even though recently ERα has been detected in the nuclei of epithelial cells of the epididymis (77). Both ERα and ERβ 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 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 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, but 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 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 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). Ejaculated human spermatozoa continue to express P450 aromatase and contain active aromatase, and sperm can 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 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.

3.5 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 ERαKO 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 ERαKO 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 ERαKO 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 ERαKO 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). Detailed investigations about the development of efferent ductules in ERαKO 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 (ERβKO) (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 ERαKO mice. In fact, unlike ERαKO mice, male ArKO mice are initially fully fertile (93), but fertility decreases with advancing age (95), and, conversely, ERβKO mice are fully fertile and apparently reproductively normal in adulthood (94). Histology of the testes of one-year-old ArKO mice shows a disruption of spermatogenesis (95). The late onset of the alterated phenotype in male ArKO mice is attribuitable to estrogenic substance present in their diet and able of agonistic effect on spermatogenesis (96). Despite the phenotype of ERαKO 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 (ERαβKO mice) showed a male phenotype very close to that of ERαKO mice with infertility and dilated seminiferous tubules (69). These findings, together with the observation that ERβKO 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 ERαKO, ERαβKO 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.

3.6 Role of estrogens in human male reproduction

The demonstration of abundant ERs in human efferent ducts and aromatase activity in human sperm, is in agreement with the hypothesis 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.

3.7 Effects of excessive estrogen on male reproduction

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

3.7.2 Aromatase over-expression in rodents

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 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).

3.7.3 Exposure to excess of 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, 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.

3.7.4 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).

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 IGFs are 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. 4.1).

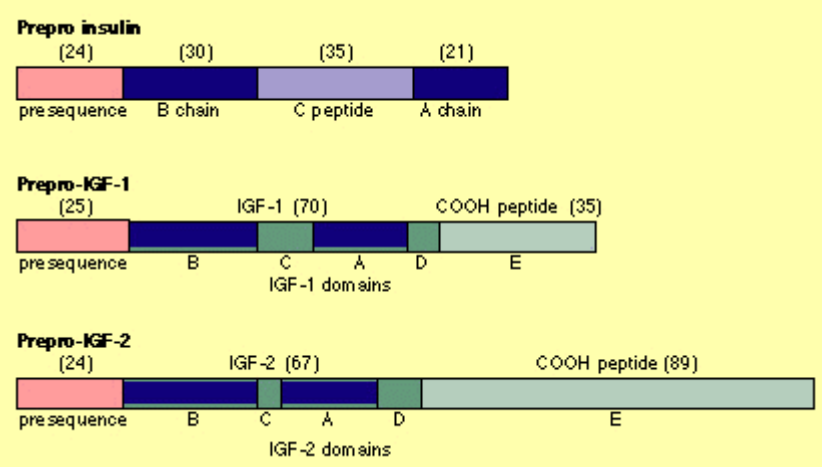


Figure 4.1. Protein domains in IGFs and Insulin. Regions showing homologies with insulin are indicated by dark blue rectangles. The lengths of individual fragments (in amino acids) are indicated in brackets.

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 (IGFIR), 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 IGFIR buth with lower affinity (161). Expression of the IGFIR 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-1-6) 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;
4) modulate, inhibit, or facilitate interaction of IGFs with their receptors (175, 176).

IGFBPs biological activities are 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 cell 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 not completly determined.

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

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). 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 (208, 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. Materials and Methods I

5.1 Cell cultures

R2C cells (a rat Leydig tumor cell line) (Fig. 5.1) were bought from American Type Culture Collection (LGC Standards, Teddington, Middlesex UK), grown for 2 weeks (four passages) before freezing aliquots. Each aliquot was used for no more than ten passages. Cells were cultured in Ham/F-10 (Sigma St Louis, MO, USA) medium supplemented with 15% horse serum (HS), 2.5% fetal bovine serum (FBS) and antibiotics (Sigma). Cell monolayers were subcultured onto 30 mm dishes for protein or RNA extraction ($1x10^6$ cells/plate), 12 well culture plates for steroid measurement ($2x10^5$ cells/well) and 24 well culture plates proliferation assay ($1x10^5$ cells/well), and used for experiments 48 h later.

Human embryonic kidney (HEK)-293 cells (Fig. 5.2) were cultured in Dulbecco's modified Eagle's/Ham F12 (DME/F12) medium (Sigma) supplemented with 5% FBS (Sigma) and antibiotics. Cell monolayers were subcultured onto 24 well culture plates for transfection experiments.

Cell cultures were treated for the indicated times with PD98059, LY294002, GF109203X (Calbiochem, VWR International S.R.L. Milano), AG1024, ICI182,780, nandrolone, stanozolol, IGF-I, 17β-estradiol, testosterone and dihydrotestosterone (Sigma).

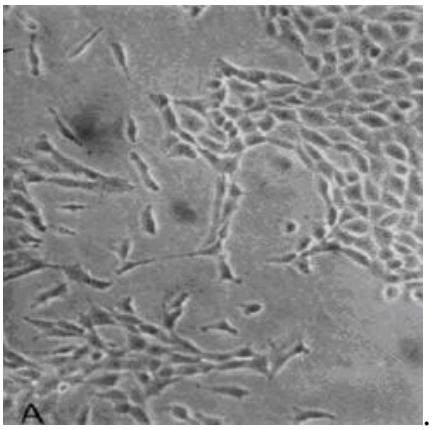


Figure 5.1. R2C cells.

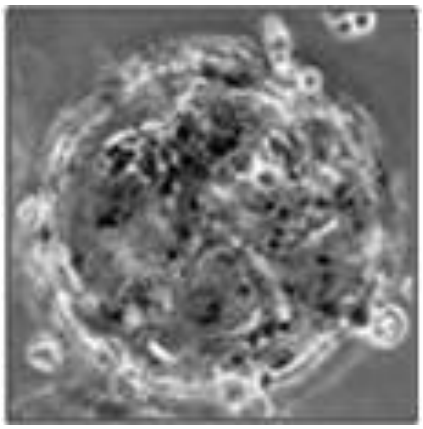


Figure 5.2. HEK-293 cells.

5.2 Radioimmunoassay

Prior to experiments, R2C cells were maintained in Ham/F-10 without serum or antibiotics (serumfree medium). Cells were then treated as necessary and estradiol content of medium recovered from each well was determined against standards prepared in serum-free medium using a radioimmunoassay kit (Diagnostic System Laboratories, Webster, TX, USA). Results were normalized to the cellular protein content per well. For IGF-I determination cells were cultured for 48h before treatment for an additional 72h in Ham/F-10 containing 1 % dextrane charcoal coated (DCC) FBS. IGF-I content in medium recovered from each well of R2C cells was determined following extraction and assay protocol provided with the rat IGF-I radioimmunoassay kit (DSL 2900; Diagnostic System Laboratories, Webster, TX, USA).

5.3 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 (164). 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 (165).

5.4 Western blot analysis

R2C cells 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 (165). 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) anti-human P450 aromatase antibody (Serotec, Oxford, UK) (1:50), (b) anti-cyclin E (M-20) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:1000), (c) anti-pIGF1R antibody (1:500; Cell Signaling

Technology, Massachusetts, USA), (d) anti-pERK antibody (1:500; Cell Signaling Technology), (e) anti-pAKT antibody (1:500; Santa Cruz Biotechnology), (f) anti-IGF1R antibody (1:500; Santa Cruz Biotechnology), (g) anti-ERK antibody (1:1000; Cell Signaling Technology), (h) anti-AKT antibody (1:1000; Santa Cruz Biotechnology), (i) anti-GAPDH antibody (1:1000; Santa Cruz Biotechnology) (1:1000). Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and immunoreactive bands were visualized with the ECL western blotting detection system (Amersham). To assure equal loading of proteins, membranes were stripped and incubated overnight with GAPDH antibody.

5.5 RNA extraction, reverse transcription and PCR

The TRizol RNA isolation system (Invitrogen S.r.l., San Giuliano Milanese (MI), Italy) was used to extract RNA from R2C cells. Each RNA sample was treated with DNase I (Ambion, Austin, TX, USA), 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 transcriptase system kit (Promega Italia S.r.l., Milano, Italy).

Samples were aliquoted and stored at -20 °C. PCR amplification was performed using 1.5 U of Taq DNA polymerase (Promega) in PCR buffer containing 200 µM dNTP, 1.5mM MgCl2, and 25 pmoles of each primer in a total volume of 50 µl. The nucleotide sequences of the primers for CYP19 were:

forward 5′-CAGCTATACTGAAGGAATCCACACTGT-3′

reverse 5′-AATCGTTTCAAAAGTGTAACCAGGA-3′.

L19 ribosomal protein mRNA was used as housekeeping gene; the nucleotide sequences of the primers for L19 were:

forward 5′-GAAATCGCCAATGCCAACTC-3′

reverse 5′-ACCTTCAGGTACAGGCTGTG-3′ .

To avoid products due to DNA contamination, primers were designed to amplify a region across different exons. The PCRs were performed for 25 cycles for P450 aromatase (94 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min) and 25 cycles for L19 (94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min).

PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide (Sigma).

5.6 Cell proliferation assay

3-[4,5-Dimethylthiaoly]-2,5-diphenyltetrazolium bromide (MTT) assay was conducted to detect cell proliferation (166). A total of 1x105 cells were seeded onto twenty-four-well plates in complete medium and let grow for 2 days. Prior to experiments, cells were maintained overnight in Ham/F-10 serum-free medium and the day after treated. There were triplicates for each concentration. Forty-eight hours after treatment fresh MTT (Sigma), re-suspended in PBS, was added to each well (final concentration 0.33 mg/ml). After 1h incubation, the culture media were discarded and replaced with 100 µl of DMSO (Sigma). The optical density was measured at 570 nm in a spectrophotometer.

5.7 Transfection assay

Before transfection, complete medium was removed, and 0.5 ml of DMEM/F12 without phenol red, serum or antibiotics was added to the plates. Transfection was performed using Fugene6 reagent (Roche Diagnostics, Mannheim, Germany), following the manufacturer's instruction. Plasmids were used at the concentration of 0.5 µg/well for the XETL (167) promoterluciferase reporter plasmid, of 0.1 µg/well for ERα expression vector (168), of 10 ng/well for the β-galactosidase control vector (Promega).

Four hours after transfection, the medium was removed and replaced with DMEM/F12 without phenol red, serum or antibiotics and supplemented with the indicated concentrations of treatments for 24 h. Cells were lysed using the passive lysis buffer (Promega), and enzymatic activities were assayed using the Luciferase (Promega) and β-galactosidase (Ambion) assay systems following the manufacturer's instructions. Firefly luciferase values of each sample were normalized by β-galactosidase activity and data were reported as relative light units (RLU) values.

5.8 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).

6.Results I

6.1 Nandrolone and stanozolol control Leydig cell proliferation through the induction of aromatase expression and estradiol production.

We first evaluated if nandrolone and stanozolol were able to induce R2C cell proliferation. Cell proliferation was measured in R2C cells treated with increasing doses of nandrolone (Fig. 6.1 A) and stanozolol (Fig. 6.1 B), revealing a significant induction with all tested doses of both androgens, with 1 μM being the most effective. Since proliferation of R2C cells depends on estrogen production (26), we evaluated the effects of nandrolone and stanozolol on aromatase expression. For this purpose R2C cells were treated for 24h with increasing doses of the two androgens and aromatase protein expression was evaluated by western blot analysis (Figure 6.1 C, D).

Both androgens were able to significantly increase aromatase levels, with maximum induction seen with 1 μM (Fig. 6.1 C, D). We also evaluated endogenous estrogen production in response to nandrolone and stanozolol, revealing the ability of both androgens to increase estradiol production as a consequence of effects on aromatase expression (Fig. 6.1 E). To confirm that proliferative effects depended on the ability of the two androgens to induce estradiol production, cells were treated with nandrolone and stanozolol in the presence of estrogen receptor antagonist ICI182,780 (ICI). As seen in figure 1F ICI reduced the effects of both androgens.

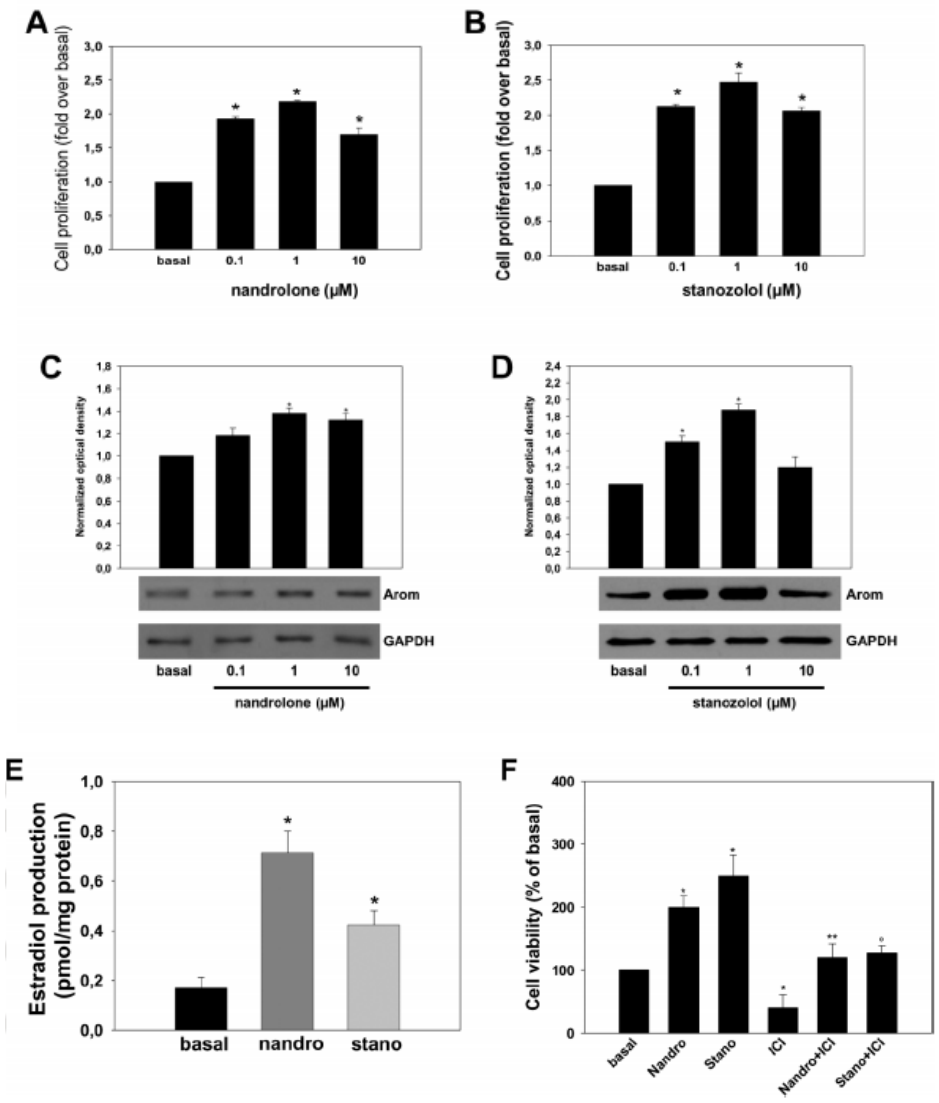


Figure 6.1: Effects of Nandrolone and Stanozolol on Aromatase expression and estradiol production in R2C cells. (A and B) R2C cells were left untreated (basal) or treated for 24h with nandrolone (nandro) (A) or stanozolol (stano) (B) at the indicated concentrations. Cell proliferation was assessed using the MTT method as indicated in the Materials and Methods section. Final results represent mean ± S.D. of three independent experiments each performed in triplicate. (*, P<0.001 compared with basal). (C and D) R2C cells were left untreated (basal) or treated for 24h with nandrolone (nandro) (C) or stanozolol (stano) (D) at the indicated concentrations. Western blot analysis for aromatase was performed on 50 μg of total proteins. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control. Graphs represent means of Aromatase optical densities normalized to GAPDH content of the same sample(*, P<0.001 compared with basal). (E) Cells were treated with nandrolone (nandro) (1 μM) or stanozolol (stano) (1 μM) for 48h. E2 content in cell culture medium, measured by RIA, was normalized to the well protein content. Values represent means ± S.D. of three different experiments, each performed in triplicate (*, P<0.001 compared with basal). (F) R2C cells were left untreated (basal) or treated for 48h with nandrolone, (nandro) (1 μM), stanozolol (stano) (1 μM) and ICI182,780 (ICI) (10 μM) alone or in combination. Proliferation was assessed using the MTT method as indicated in the Materials and Methods section. Final results represent mean ± S.D. of three independent experiments. (*, P<0.001 compared with basal; **, P<0.001 compared with nandro; °, P<0.005 compared with stano).

6.2 Nandrolone or stanozolol in combination with IGF-I further induce Leydig cell proliferation and aromatase expression.

Since we have previously shown a role for IGF-I in Leydig cell proliferation (26), we also evaluated effects of combined treatments of IGF-I with nandrolone or stanozolol on cell proliferation in R2C cells (Fig. 6.2). The dose of 1 μM was used in combination with IGF-I (100 ng/ml) and cell proliferation was measured (Fig. 6.2 A). Results obtained show that both nandrolone and stanozolol have an additive effect with IGF-I in inducing proliferation (Fig. 6.2 A). Also in this case, the estrogen antagonist ICI was able to significantly reduce both AAS- and AAS+IGF-Idependent proliferative effects (Fig. 2A). An additive effect in inducing aromatase expression was found when IGF was combined with nandrolone or stanozolol (Fig. 6.2 B, C). Data obtained on aromatase expression were also mirrored by effects on aromatase activity (Fig. 6.2 D). IGF-I receptor (IGF1R) activation by direct ligand binding or by indirect transactivation determines activation of three major transductional pathways: Ras/Raf/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/AKT, and phospholipase C (PLC)/protein kinase C (PKC). We tested the effects on cell proliferation of specific inhibitors such as PD98059 for MAPK; LY294002 for PI3K/AKT, (LY), GF109203X (GFX) for PLC/PKC and AG1024 (AG) for IGF1R, used in combination with nandrolone or stanozolol (Fig. 6.3 A). The use of AG, LY and GFX was able to reverse nandrolone and stanozolol-induced cell proliferation (Fig. 6.3 A). PD was the only inhibitor that did not interfere with cell proliferation. To demonstrate the link between cell proliferation and estrogen production we evaluated the effects of the inhibitors on aromatase expression (Fig. 6.3 B-D). AG, LY and GFX were able to reduce nandrolone- and stanozolol-induced aromatase mRNA (Fig. 6.3 B) and protein (Fig. 6.3 C, D) levels. The ability of inhibitors for IGF-I-dependent pathway to control nandrolone and stanozolol effects led us to suppose that the two AAS could regulate IGF-I production in R2C cells. Basal IGF-I content in R2C cell culture medium was 80 mg/ml/mg protein and was increased by 2- and 3.4-fold by nandrolone and stanozolol, respectively (Fig. 6.4 A). When IGF-I was neutralized with a specific antibody cell proliferation was markedly decreased (Fig. 6.4 B). Nandrolone and stanozolol could not overcome the inhibitory effect produced by the antibody (Fig.

6.4 B). These data confirm that R2C proliferation is tightly dependent on IGF-I and indicate that a mechanism through which nandrolone and stanozolol control R2C proliferation is through the induction of IGF-I production.

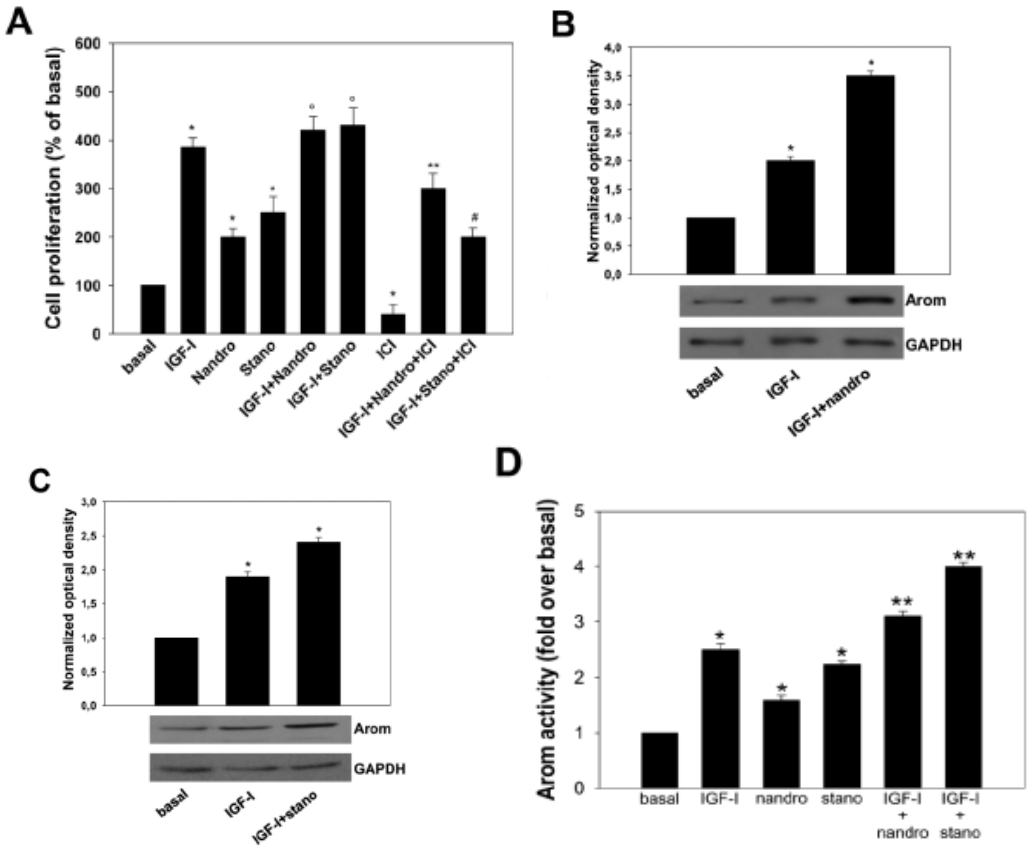


Figure 6.2: Additive effects of nandrolone or stanozolol and IGF-I on cell proliferation and aromatase expression and activity. (A) R2C cells were left untreated (basal) or treated for 48h with IGF-I (100 ng/ml), nandrolone (nandro) (1 μM), stanozolol (stano) (1 μM) and ICI182,780 (ICI) (10 μM) used alone or in combination. Cell proliferation was assessed using the MTT method as indicated in the Materials and Methods section. Final results represent mean ± S.D. of three independent experiments each performed in triplicate. (*, P<0.001 compared with basal; °, P<0.001 compared with IGF-I; **, P<0.005 compared with IGF-I+nandrolone; # P<0.001 compared with IGF-I+stano). (B and C) R2C cells were left untreated (basal) or treated for 24h with IGF-I (100 ng/ml) used alone or in combination with nandro (1 μM) (B) or stano (1 μM) (C). Western blot analysis for aromatase was performed on 50 μg of total proteins. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control. Aromatase optical densities were normalized to GAPDH content of the same sample. Graphs represent means of values from three blots, where basal values were assumed as 100. (*, P<0.001 compared with basal). (D) R2C cells were left untreated (basal) or treated for 48h with nandro (1 μM) or stano (1 μM) and IGF-I (100 ng/ml) alone or in combination. Aromatase activity was assessed using the modified tritiated water method. Result obtained were measured in pmoles of released $^{[3H]}H20$, normalized to the well protein content (pmol/mg protein/h) and expressed as fold over basal. Values represent the mean ± S.D. of three independent experiments each performed in triplicate (*, P<0.001 compared with basal; **, P<0.001 compared with IGF-I).

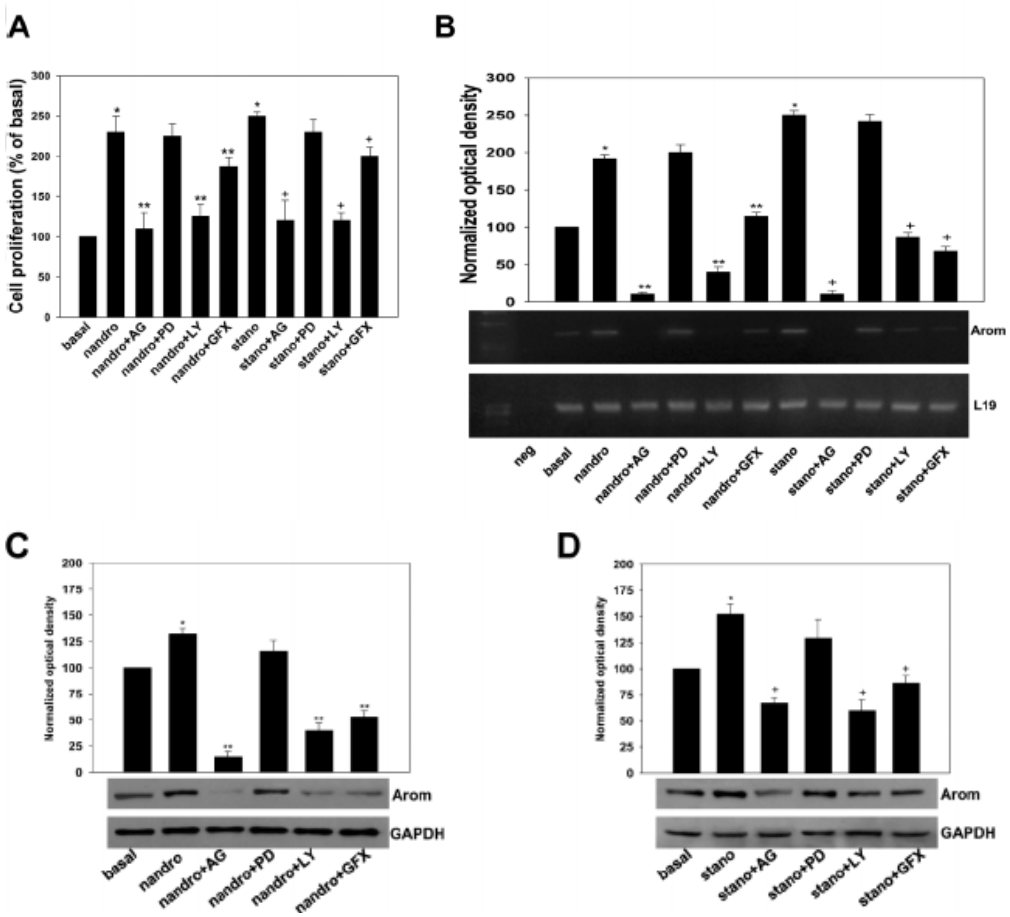


Figure 6.3: Effects of IGF-I pathway inhibitors on nandrolone and stanozolol induced cell proliferation and aromatase expression. (A-D) R2C cells were left untreated or treated in serum-free medium for 48h (A) or 24h (B-D) with nandrolone (nandro) (1 μM) or stanozolol (stano) (1 μM) alone or in combination with AG1024 (10 μM) (AG), LY294002 (10 μM) (LY), PD98059 (10 μM) (PD) and GF109203X (GFX) (10 μM). (A) Cell proliferation was assessed using the MTT method as indicated in the Materials and Methods section. Final results represent mean ± S.D. of three independent experiments each performed in triplicate. (*, P<0.001 compared with basal; **, P<0.001 compared with nandrolone; +, P<0.001 compared with stanozolol). (B) Total RNA was extracted from cells untreated (basal) and treated as indicated. RT-PCR was used to analyze mRNA levels of CYP19. L19 was used as housekeeping gene. Negative control (neg) was obtained using water instead of cDNA. Image is representative of three independent experiments with similar results. (C and D) Western blot analysis for aromatase was performed on 50 μg of total proteins.
Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control. Aromatase optical densities were normalized to GAPDH content of the same sample. Graphs represent means of values from three blots, where basal values were assumed as 100. (*, P<0.001 compared with basal; **, P<0.001 compared with nandrolone; +, P<0.001 compared with stanozolol).

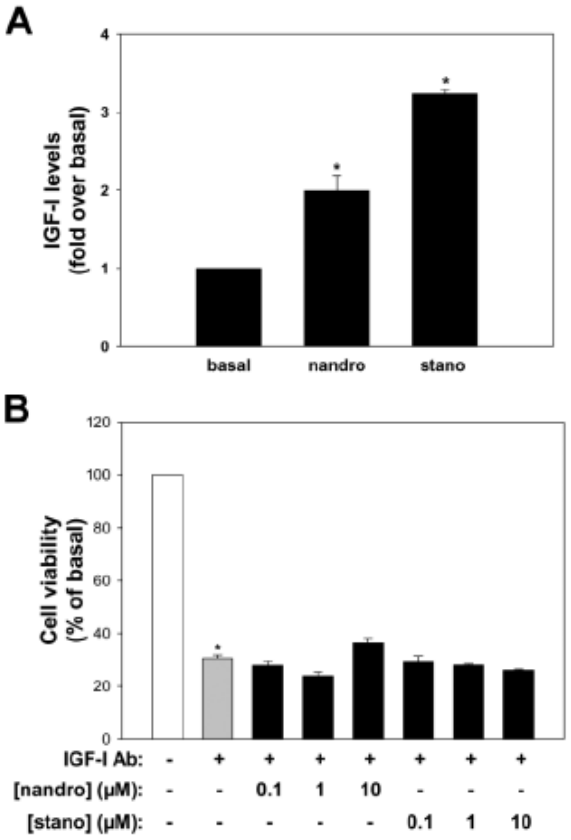


Figure 6.4: Nandrolone and stanozolol effects on cell proliferation depend on IGF-I production. (A) R2C cells were left untreated (basal) or treated for 48h with nandrolone (nandro) (1 μM) or stanozolol (stano) (1 μM) in 1% DCC. IGF-I levels in culture medium were determined by RIA and IGF-I content was normalized to the cell culture well protein content. Data represent the mean ± SEM of values from three separate cell culture wells expressed as fold over basal. (*) P< 0.01 compared with basal conditions. (B) Cell proliferation was assessed using the MTT method as indicated in the Materials and Methods section. IGF-I antibody (IGF-I Ab) was added to the medium at 5 μg/ml 24h before being treated for an additional 24h with the indicated concentrations of nandrolone and stanozolol. Columns, mean percent of untreated (basal) cells (100%) from three independent experiments each done in triplicate; bars, S.D. *, P < 0.01 compared with basal.

6.3 Nandrolone and stanozolol activate rapid signaling through Erα transactivation.

Recently it has been demonstrated that androgens can activate alternative pathways, in addition to classic genomic mechanisms of regulation of gene expression (169). For this reason we decided to investigate the ability of nandrolone and stanozolol to activate rapid signalling involving IGF1R phosphorylation and activation of kinases involved in cell proliferation. R2C cells were treated for 10 minutes with increasing doses of the two androgens and IGF1R, ERK and AKT phosphorylation was

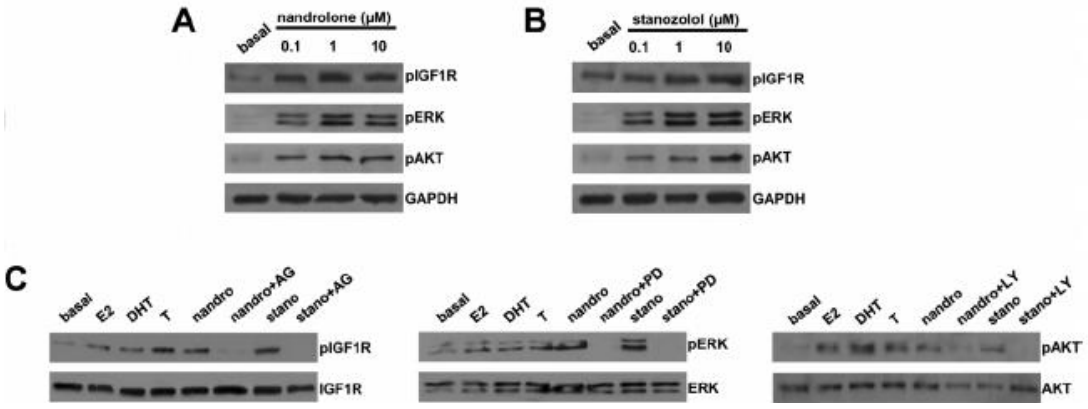
evaluated by western blot analysis. Both androgens were capable to increase kinase activity at all used doses (Fig. 6.5 A, B).

The effects produced by the two AAS on the activation of IGF1R-dependent pathways were reproduced also by E2, dihydrotestosterone (DHT) and testosterone (T) (Fig. 6.5 C). In addition the presence of specific inhibitors for IGF1R, ERK1/2 and AKT were able to prevent kinases activation (Fig. 6.5 C).

There are numerous reports suggesting that androgens might act through ERα by themselves (170) and our data shown in Figure 1F and 2A indicate that this could also be the case for the two AAS in R2C cells.

To define if the rapid AAS-dependent signaling was mediated by ER, R2C cells were treated with the two androgens in the presence of the estrogen receptor antagonist ICI182,780. As shown in Figure 5D ICI administration reduced the effect of both androgens on IGF1R phosphorylation.

To evaluate the specificity of nandrolone and stanozolol to transactivate ERα we performed cotransfection experiments using a luciferase reporter plasmid (XETL), a construct containing an estrogen-responsive element (167), and the human ERα expression vector (HEGO) (168). To avoid interference by endogenous estrogen receptors transfections were performed in HEK293 cells that do not express ERs. Eighteen hours after transfection cells were exposed to increasing concentrations of the two androgens. Our results demonstrate the ability of both androgens to transactivate ERα (Fig. 6.5 E).



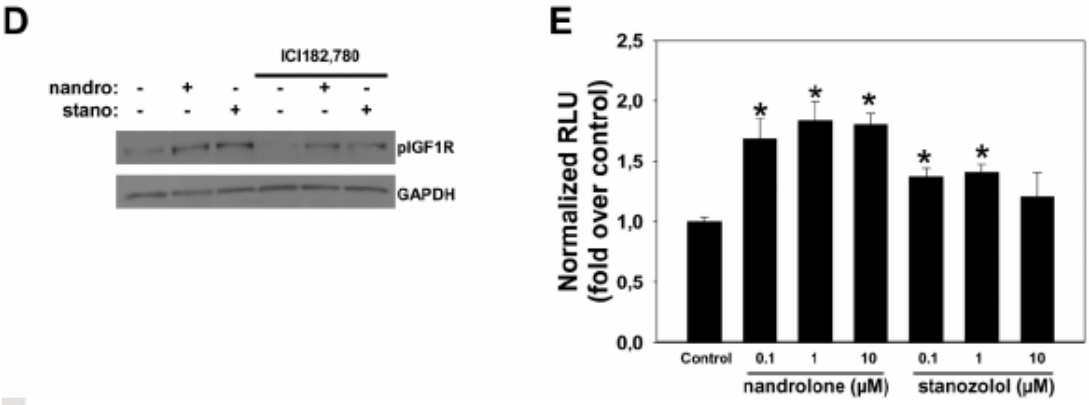


Figure 6.5: Effects of nandrolone or stanozolol on ER-activated IGF1R signalling. R2C cells were left untreated (basal) or treated for 10 min with nandrolone (nandro) (A) or stanozolol (stano) (B) at the indicated concentrations. (C) R2C cells were left untreated (basal) or treated for 10 min with E2 (100 nM), dihydrotestosterone (DHT) (100 nM), Testosterone (T) (100 nM), nandrolone (1 μM), stanozolol (1 μM) combined with AG1024 (10 μM) (AG), PD98059 (10 μM) (PD) and LY294002 (10 μM) (LY). (D) R2C cells were left untreated (basal) or treated for 10 min with nandrolone (1 μM), stanozolol (1 μM) and ICI182,780 (ICI) (10 μM) used alone or in combination. Western blot analysis was performed on 50 μg of total proteins. Blots are representative of three independent experiments with similar results. GAPDH (A, B, D) or alternatively IGF1R, ERK1/2 and AKT (C) were used as loading control. (E) HEK293 cells were transiently transfected using XETL reporter plasmid and ERα expression vector and treated with the indicated doses of nandrolone or stanozolol. Data were normalized to the coexpressed β-galactosidase expression vector and expressed as RLU. Results represent the mean + S.D. of data from three independent experiments, each performed in triplicate. (*, P<0.001 compared with basal).

6.4 Nandrolone and stanozolol influence cell proliferation by increasing cyclin E expression levels.

To explain the observed effects on cell proliferation we evaluated expression of cyclin E, that we have previously shown to be up-regulated by IGF-I in R2C cells (26). Cyclin E protein expression was increased by all used doses of both nandrolone (Fig. 6.6 A) and stanozolol (Fig. 6.6 B). In addition, combined treatments with IGF-I and either steroid, caused a stronger induction of cyclin E expression (Fig. 6.6 C, D).

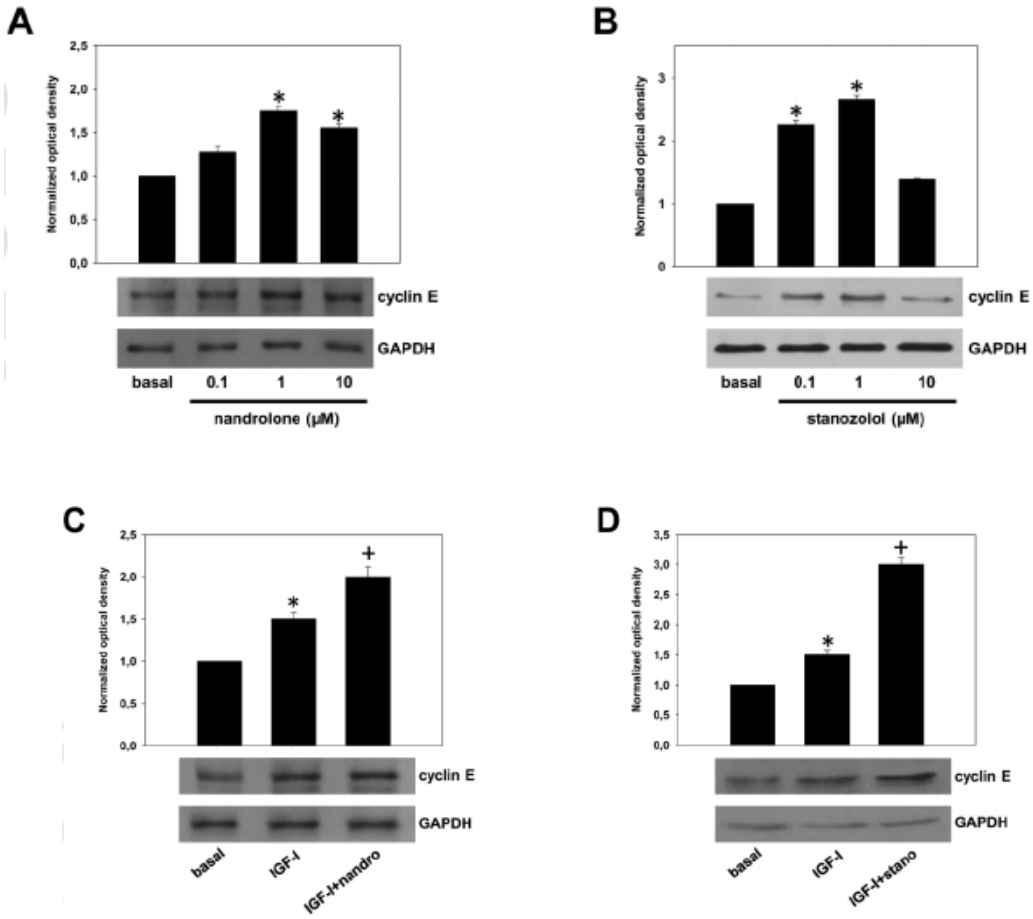


Figure 6.6: Additive effects of nandrolone or stanozolol and IGF-I on cyclin E expression in R2C cells. R2C cells were left untreated (basal) or treated for 24h with nandrolone (nandro) (A) or stanozolol (stano) (B) at the indicated concentrations, nandrolone (nandro) (1 μM) plus IGF-I (100 ng/ml) (C) or stanozolol (stano) (1 μM) plus IGF-I (100 ng/ml) (D). Western blot analysis for cyclin E was performed on 50 μg of total proteins. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control. Cyclin E optical densities were normalized to GAPDH content of the same sample. Graphs represent means of values from three blots, where basal values were assumed as 100. (*, $P<0.001$ compared with basal; +, $P<0.001$ compared with IGF-I).

7. Discussion I

Leydig cells are the main site of testosterone biosynthesis in the male mammals, as well as a site of conversion of testosterone to estradiol through aromatase activity (171). Alterations in local estrogen synthesis may have significant consequences in malignancy of these cells. In a previous study, we observed that R2C tumor Leydig cells release a conspicuous amount of E2, significantly higher than normal Leydig cell cultures, as a consequence of aromatase overexpression in tumor cells (26). It can be suggested that in the presence of increased androgens availability, their metabolism through aromatase, expressed in Leydig cells, increases local estrogen levels, contributing to the initiation or progression of Leydig cell tumor. We tested the effects of two commonly used AAS nandrolone (aromatizable) and stanozolol (nonaromatizable) and evaluated the effects on aromatase expression and Leydig cell proliferation. Since muscle mass and strength are correlated with the administered dose and the circulating concentrations of AAS (172, 173), the doses commonly used in practice are extremely high and range from 500 to 1000 mg/weekly (2, 3). These observations support our decision to test the effects of high doses (0.1 to 10 μM) of nandrolone and stanozolol. When we evaluated the effects of these two androgens on aromatase expression we found that they are both able to increase the enzyme protein levels and consequently estrogen production in R2C cells, with stanozolol being more effective in inducing the enzyme but nandrolone more effective in increasing estrogen levels. A plausible explanation for this behavior is that nandrolone is immediately metabolized to estradiol by R2C cells, where aromatase is constitutively active, and the amount left to activate the mechanism responsible for increasing aromatase transcription is less than the concentration effectively used to treat cells. On the other side, stanozolol is not metabolized by aromatase, and the concentration used to treat cells is effectively bio-available to determine the effects on aromatase transcription. Thus, both AAS contribute to estrogen production determining an effect on tumor cell proliferation that we could define “indirect”, not related to their androgenic properties, but estrogen-dependent. This effect is

confirmed by the observation that ICI reduces AAS-dependent cell proliferation. However, since we found that treatments with inhibitors for IGF1R, PKC and PI3K

were able to reduce AAS effects on aromatase expression and cell proliferation, it could also be suggested that these molecules directly activate non-genomic hormonal signals. We supposed that the two AAS could activate estrogen receptor (ER)-dependent pathways. The estrogenic actions of androgens are established (174), and the possibility for AAS to activate ERs is consistent, also in our experimental system, with the ability of ICI to reduce AAS-dependent cell proliferation. In addition, AAS acting on a membrane bound ER activate IGF1R and downstream kinases, as seen in several cell systems (145). These events could partially explain the inhibition produced by AG1024, the IGF1R inhibitor, and by downstream IGF-I signaling inhibitors (GFX for PKC and LY for PI3K). The use of high doses of AAS could cause in our cell system ER/AR desensitization, explaining the reduced effects seen with 10 μM of both nandrolone and stanozolol. Our results give interesting indications and open several hypothesis on possible non genomic molecular mechanisms induced by AAS involving IGF-I dependent signaling pathways. For example, GPR30, a novel estrogen receptor (152), could also be activated by AAS, explaining why ICI was not able to completely block nandrolone- and stanozolol-dependent IGF1R activation.

Additionally, the two AAS can also work through a membrane AR to activate IGF1R signaling.

It was shown for human primary prostatic stromal cell cultures, that administration of DHT and T, but not of E2, modulated IGF-I protein expression (175). In our cell model the two androgens induce IGF-I production, and when this growth factor was immunoneutralized, cell proliferation was deeply reduced and nandrolone and stanozolol lost their ability to increase cell proliferation, confirming our previous report indicating IGF-I as indispensable for R2C cell proliferation (26). In addition, the observation that stanozolol was more effective than nandrolone in inducing IGF-I production, supports the hypothesis that nandrolone metabolism to estradiol reduces its bio-available amount.

IGF-I signalling is highly involved in cancer development and progression (176) exerting powerful effects on each of the key stages of cancer formation: cellular proliferation, apoptosis, angiogenesis, metastasis and resistance to chemotherapeutic agents. We previously showed that IGF-I, endogenously produced by Leydig tumor

cells, activating PI3K/AKT and PLC/PKC (but not MAPK) pathways determines an increase in aromatase expression and estrogen production inducing cell proliferation (26). In the current study we confirmed these data and tested also the effect of combined treatments of nandrolone or stanozolol with IGF-I. Effects of the two AAS were potentiated by the presence of IGF-I. The evaluation of aromatase activity revealed that the combined treatments of nandrolone or stanozolol with IGF-I increased the enzyme activity to levels above those seen with the single treatments. All the effects seen on aromatase were mirrored by changes in R2C proliferative behavior; nandrolone and stanozolol increase cell proliferation and their effects are additive with IGF-I. One mechanism through which estrogens and IGF-I induce cell proliferation is by increasing protein levels of G1 regulatory cyclins A, B1, D1, D3, and E in target cells (177, 178). In our study, we showed that the expression of one of the most important regulators of Leydig cell cycle, cyclin E, can be increased by nandrolone and stanozolol and significantly increased to a greater extent by the combined treatment with IGF-I. These result further confirm that AAS could contribute to activate expression of estrogen- and IGF-I-dependent genes involved in cell cycle progression.

In conclusion, despite the growing body of data over the past decade that has established that androgens increase muscle mass (172) in correlation with the administered dose and the circulating concentrations (173), it should be seriously taken into account the potential dangerous effects produced by the use of AAS on the activation of pathways involved in the progression of a type of cancer, such as Leydig cell tumor, with high incidence in young people, the same age people abusing AAS. In this study we have shown that aromatizable and nonaromatizable androgens can promote testicular tumor development through the induction of aromatase expression, estrogen and IGF-I production. In addition, the two tested androgens binding ERα transactivated IGF1R activating PI3K and PKC pathways determining an induction of R2C tumor cell proliferation. The examined AAS effects are potentiated by the concomitant use of IGFI.

It would be interesting to determine the effect of the two AAS in human cultures of Leydig cells, that, however are currently not available, as well as *in vivo* in an animal model. Before beginning any illegal and self-determined use of doping agents deep

consideration should be given to the deleterious effects associated with their use, among which we can now include Leydig cell tumor.

Rationale II

Adrenocortical tumors (ACTs), mostly benign adenomas (ACA), are frequent in the general population and nowadays are most often found incidentally (179). By contrast, adrenocortical carcinoma (ACC) is rare, with an estimated prevalence of 4-12 per million in adults. The prognosis of ACC is very poor, with a 5-year survival rate below 35% in most series (180). ACC has been characterized as having a bimodal age distribution with an increased incidence in the first and fifth decades of life. Approximately 50% of ACC in adults and 90% in pediatric patients are functioning and produce hormonal and metabolic syndromes leading to their discovery (181). The cause of adrenal cancer remains elusive, but studies in the past 10 years suggest genetic mutations in the adrenal gland leading to the initiations of a malignant tumor (180, 182, 183). Somatic mutations of the tumour suppressor gene TP53 are observed in a third of ACC. Interestingly, allelic losses (LOH) at the TP53 locus (17p13) are very frequent and observed in more than 85% of ACC (184). Transcriptome analysis suggests also that the Wnt/beta-catenin signalling pathway is activated in ACT. About a third of ACC harbours somatic activating mutations of the betacatenin gene (184, 185). The most consistent and dominant genetic changes in ACC is the perturbation of the insulin-like growth factor II (IGF-II) locus (11p15) that is imprinted. IGF-II is over-expressed in 90% of ACCs determining an autocrine mitogenic effect. The direct involvement of IGF-II/IGF-IR system in adrenocortical tumor cell proliferation has been also shown in vitro using adrenal cancer cell line NCI H295R (185). Moreover, increased levels of the IGF-IR have been found in advanced human ACC, suggesting an important role for the IGF system in adrenocortical carcinogenesis (186). For this reason inhibitors for IGF-IR are currently in preclinical trials. However, ACC is a disease extremely heterogeneous and this new pharmacological approach could not be enough for the therapy of all forms of ACC, since several molecular mechanisms trigger ACC development. Thus, progress in the understanding of the pathophysiology of ACC is important to improve diagnosis, prognostic evaluation and treatment of different types of ACC. The majority of currently studies has analyzed only single pathways of signal

transduction, but it is becoming clear that ACC pathogenesis involves integration of signals and the interplay of downstream pathways.

Epidemiological and experimental studies suggest that estrogens could also be involved in genesis and progression of ACC. A type of ACC is named feminizing because secrete estrogens causing gynecomastia and testicular atrophy in man. Moreover, usually ACC are more frequent in women than in men, especially in those exposed to estro-progestin (187, 188). Professor Pezzi's group have demonstrated that ACC are characterized by ERα up-regulation and aromatase (the enzyme involved in the production of estrogens using androgens as substrate) over-expression (189) and that estradiol enhances proliferation of the human adrenocortical carcinoma cell line H295R, whereas antiestrogens upregulate ERβ and inhibit ACC cell growth (190). Recently, it has been demonstrated by immunohistochemistry the presence of 17β-hydroxysteroid dehydrogenase type 5 and aromatase (enzymes required to synthesize 17β-estradiol) in a feminizing adrenal cortical carcinoma (191). In the same study it has been shown that H295R cells can synthesize 17β-estradiol directly from cholesterol suggesting the presence of an autocrine loop in feminizing adrenal cortical carcinoma cells, in which estrogens are used to induce proliferation. Furthermore emerging evidences suggest that ER mediated genomic, as well as nongenomic, signalling events require functional interactions with co-regulators (192). Among several ER co-regulators, proline-, glutamic acid-, leucine-rich protein-1 (PELP1) (193) (also previously known as modulator of the nongenomic actions of the estrogen receptor, MNAR (194)) is unique because it plays important roles in both genomic (195) and nongenomic actions of the ER (196), (197). In fact, PELP1 functions as a coactivator of both ERα (193) and ERβ (198) and modulates their transactivation functions at genomic level. Moreover, PELP1 participates in ER cytoplasmic and membrane-mediated signaling (MIS) events by coupling the ER with several cytosolic kinases acting as a scaffolding protein and facilitating activation of ER-nongenomic signaling pathways (146). PELP1 modulates the interaction of ERs with cSrc, stimulating cSrc enzymatic activity, leading to the activation of the mitogen activated protein kinase (MAPK) pathway (194), but it can also directly interact with the p85 subunit of PI3K and enhance PI3K activity (197). PELP1 signaling plays an important role in E2-mediated cell cycle progression (199),

(200), in fact PELP1 is a Retinoblastoma (pRb)-interacting protein and PELP1 deregulation is shown to promote cyclin D1 expression (200). Several lines of evidence implicate PELP1 as a potential proto-oncogene and its expression is deregulated in a wide variety of hormone-driven tumors including breast (193), (201-203) endometrial (198), ovarian (204) and prostate(205) cancers. Recent studies in breast tumors suggested that PELP1 contributes to increased expression of aromatase and local E2 synthesis and that it cooperates with growth factor signaling components in the activation of the aromatase gene. PELP1 regulation of aromatase represents a novel mechanism for *in situ* estrogen synthesis, leading to tumor proliferation by an autocrine loop. Moreover, growth factors promote tyrosine and serine phosphorylation of PELP1 (197) and it has been demonstrated that PELP1 can directly interact with several growth factor receptors, including EGFR and HER2 (197)-(146), (206). Such regulatory interactions of PELP1 have important functional implications in the cross-talk of estrogen receptor and growth factor signaling (207). Aim of this project was to elucidate the role of estrogen signaling and its cross-talk with IGF system in adrenocortical tumor cell proliferation. Moreover, once we defined where the two signaling pathways converge, we focused our attention on the role played in this context by PELP1 and on the effects determined on adrenocortical tumor cell proliferation by PELP1 silencing. This approach allowed us to determine master regulators at the convergence point of different pathways, which can be targeted in a more specific pharmacological therapy compared to those currently used or used in trials.

Background II

8. Human adrenal gland

8.1 The adrenal gland: general structure

The adrenal glands (also known as suprarenal glands) are endocrine glands that lie immediately above the kidneys on their posteromedial surfaces. Human adrenal glands are triangular shaped and they are found at the level of the 12th thoracic vertebra (Figure 8.1). Each adult adrenal weighs approximately 4 g and is 2 cm wide, 5 cm long and 1 cm thick (208). The adrenal gland is a major hormone-secreting organ composed of two functionally distinct compartments: the cortex, that produces cortisol, aldosterone and sex steroids and accounts about for 90% of its volume, and the medulla, which occupies the central portion of the gland and accounts for 10% of its volume.

These two compartimens also differ for their color: yellow for the cortex and red for the medulla in relation to the abundance in blood. Adrenomedullary chromaffin cells are derived from the neuroectoderm and synthesise catecholamines that are acutely secreted in response to stress through sympathetic stimulation.

Adrenocortical cells, on the other hand, are of mesodermal origin and synthesise steroid hormones that regulate body homeostasis and mediate chronic stress responses, as part of the endocrine hypothalamic-pituitary-adrenal (HPA) axis and renin-angiotensin system.

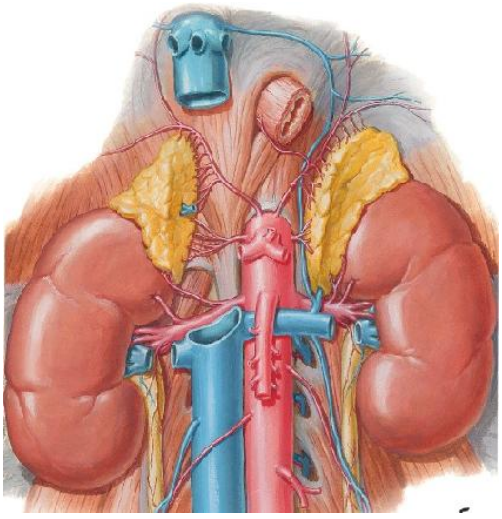


Figure 8.1 Human adrenal glands.

8.2 Embryology and development

Human adrenal development is a continuous process that initiates around the fourth week of gestation and extends into adult life. The adrenal cortex derives from the intermediate mesoderm while adrenal medulla derives from ectoderm (209).

Cells destined to become adrenal tissue migrate dorsally towards the upper pole of the mesonephros from 5 to 7 weeks of gestation. At around eight weeks gestation the adrenal primordium is formed, consisting of an inner zone of large polyhedral eosinophilic cells, termed fetal zone, surrounded by a densely packed outer zone of basophilic cells, termed definitive zone.

The primary adrenal gland is wrapped in a thick layer of compact cells that will form the adrenal cortex. From 10-12 weeks gestation on, the morphology of the developing adrenal cortex does not change much. About between 16th and 20 th week of gestation the fetal zone predominates and occupies 80 to 90% of cortical volume (Figure 8.2). Events unfolding during late embryonic and fetal stages of adrenal development are crucial for the formation of the organ and result in rapid growth of the gland, to a relative size many fold higher than that of the adult adrenal (210).

The anatomical relationships between the fetal zone and definitive zone are maintained until birth, when the fetal zone gradually disappears, reducing the weight of the adrenal cortex in the space of three months after childbirth.

Despite the adult adrenal cortex develops from the definitive zone of fetal adrenal glands, the glomerular and fascicular zones are not fully developed until the third year of age and zona reticularis may not be fully differentiated until the age of 15 years (211).

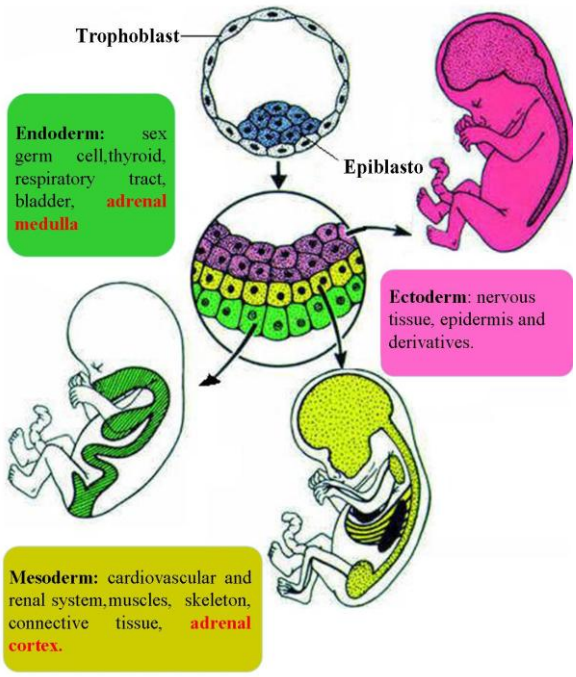


Figure 8.2 Embriogenesis.

8.3 Histology

It's possible to distinguish three histologically and functionally different concentric zone within human adrenal gland cortex: the outer *zona glomerulosa*, lying immediately below the capsule and corresponding to approximately 15% of cortical volume characterized by cells organised in rounded clusters around capillary coils or glomeruli; the middle *zona fasciculata*, corresponding to up to 75% of cortical volume, characterized by cells arranged in radial rows separated by trabeculae and by blood vessels and the inner *zona reticularis* that lies next to the medulla, in which cells are located within a uniform reticular net of connective tissue and blood vessels (212) (Figure 8.3).

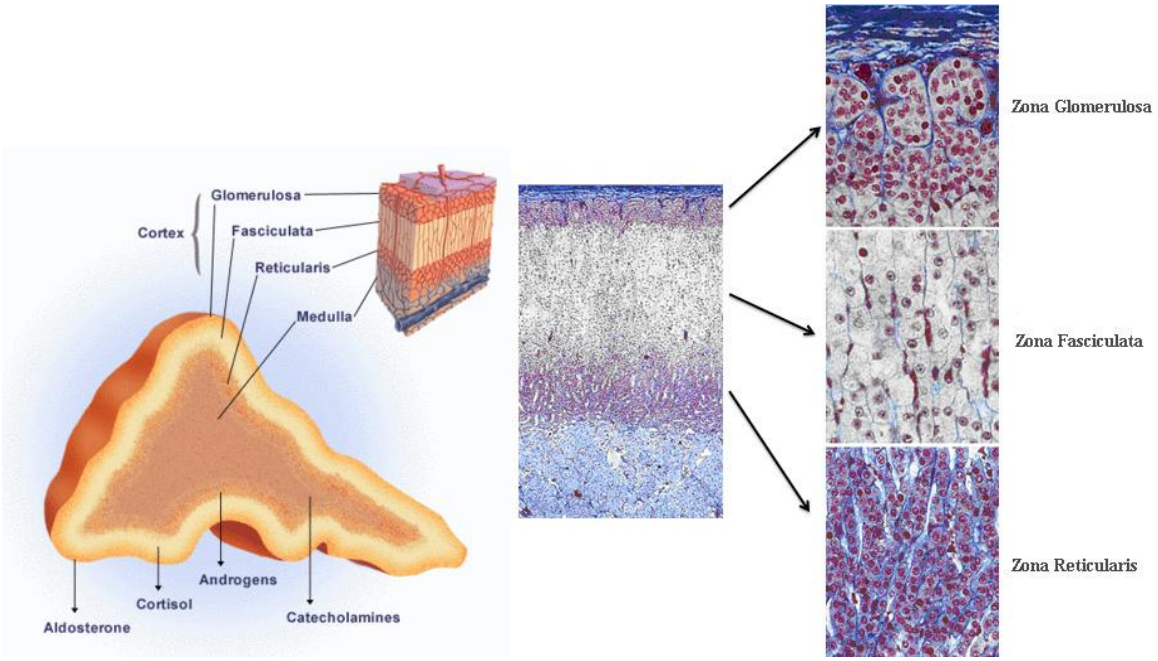


Figure 8.3 Human adrenal gland zones.

Zona glomerulosa, fasciculata and reticularis secrete different classes of steroid hormones in response to individual regulatory mechanisms. Zona glomerulosa secretes mineralcorticoids such as aldosterone under the renin-angiotensin system and potassium serum levels control. Zona fasciculata secretes glucocorticoids such as cortisol and corticosterone in response to adrenocorticotropin hormone (ACTH) (213), wile zona reticularis synthesises the sex steroid precursors dihydroepiandrosterone (DHEA), dihydroepiandrosterone sulfate (DHEAS) and androstenedione that can be peripherally converted to testosterone (214).

Remarkably, this functional zonation of the adrenal cortex is largely dependent on the zonal pattern of expression of specific enzymatic activities required to produce these different classes of steroids.

8.4 Adrenocortical steroidogenesis

Different structures are involved in steroid hormones production, such as adrenal cortex, theca and luteal cells in the ovary, and in Leydig cells in the testis and the variability in the relative expression of steroidogenic enzymes determines the types of steroids preferentially produced in each of these steroidogenic tissues.

Steroidogenesis starts from cholesterol and involves cytochrome P450 steroid hydroxylases and hydroxysteroid dehydrogenases (Figure 8.4).

Adrenocortical cells can synthesise cholesterol *de novo*, but most of their cholesterol supply comes from the uptake of plasma lipoproteins, largely low-density lipoproteins (LDL) in humans (212). In particular adrenal cortex uses cholesterol deriving from LDL through a pathway mediated by receptors whose number is enhanced by ACTH, that is also able of enhacing cholesterol esterase activity (215). Free cholesterol resulting from LDL metabolism can be readily used or esterified and stored in lipid droplets.

Most of the enzymes necessary for steroid hormones biosynthesis are members of the superfamily of cytochrome P450 (216), a group of mixed function oxidase, which play an important role in both the metabolism of xenobiotics such as drugs and environmental pollutants and in the biosynthesis of endogenous compounds such as steroid hormones, vitamin D, bile acids, fatty acids, prostaglandins and biogenic amines.

All enzymes in this family are able to reduce the atmospheric oxygen with electrons donated by NADPH according to the reaction:

$$NADPH + H^+ + O_2 + RH \xrightarrow{\textbf{P450}} R–OH+H_2O+NADP^+$$

Steroidogenesis requires the involvment of six different P45o enzymes: P450scc, the enzyme that cleaves the side chain of cholesterol and catalyzes a series of reactions formally defined 20, 22 desmolasi. Located in adrenal mitochondria, is present in two forms, the $P450_{C11}$ (11-β-hydroxylase) and $P450_{C18}$ (aldosterone synthase), which catalyze the activity of 11-hydroxylase. $P40_{C18}$ has 18-hydroxylase and 18-methoxi-activities while $P450_{C17}$, localized in the endoplasmic reticulum, catalyzes the 17-α-hydroxylase and 17, 20-lyase activities, the 21-hydroxylation of glucocorticoids and mineralcorticoid is instead catalyzed by $P450_{C21}$. In gonads and in adrenal gland the $P450_{AROM}$, present in the endoplasmic reticulum, catalyzes the reaction of aromatization of androgens into estrogens.

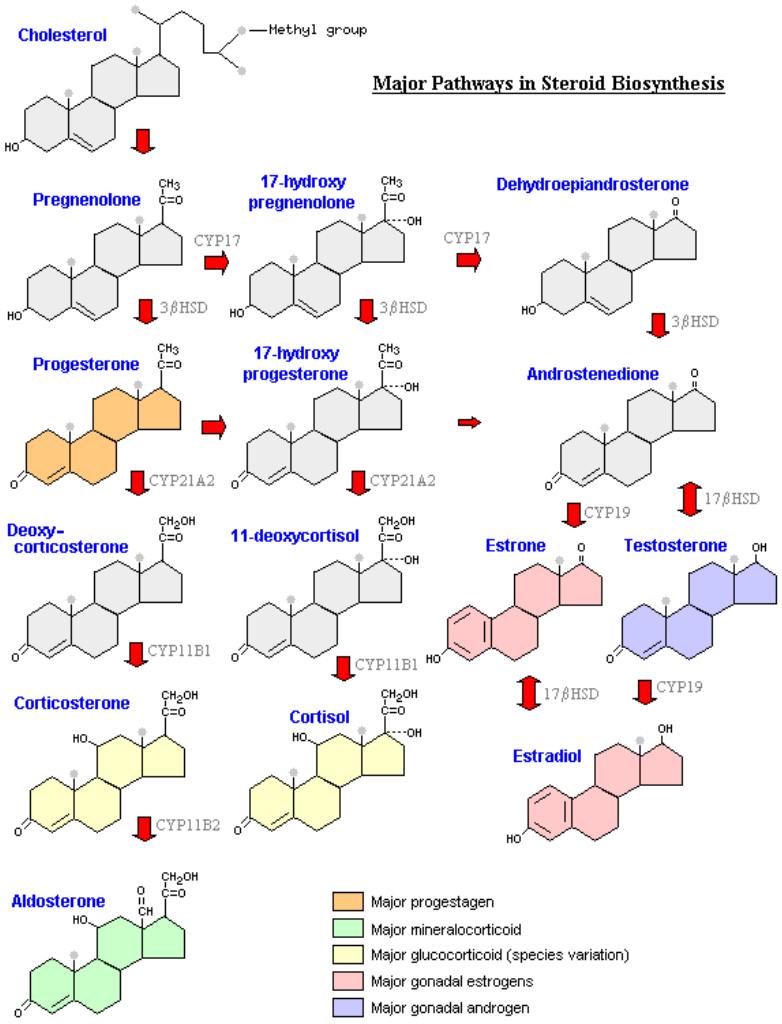


Figure 8.4 Pathway of steroid biosynthesis in the adrenal cortex. The figure shows the structure of mineralocorticoids, glucocorticoids and adrenal androgens and their precursors. The enzymes involved in the synthesis are represented on the arrow.

8.5 Adrenocortical steroidogenesis: enzymes involved

8.5.1 The StAR protein

For steroidogenesis, free cholesterol transport across mitochondrial membranes into the mitochondria is facilitated by the steroidogenic acute regulatory protein (STAR). 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 (72). It has been suggested that the protein StAR put directly

in contact, in the mitochondria, the inner membrane with the external one, allowing the passage of cholesterol, according to the concentration gradient (217).

8.5.2 $P450_{SCC}$

The initial and rate-limiting step in the pathway leading from cholesterol to steroid hormones is the cleavage of the side chain of cholesterol to yield pregnenolone. This step is catalysed by the inner mitochondrial membrane bound cholesterol side chain cleavage enzyme (P450scc, CYP11A1) (218), and involves three distinct chemical reactions: 20α-hydroxylation, 22-hydroxylation, and scission of the cholesterol side-chain to yield pregnenolone and isocaproic acid (219).

In human adrenal gene transcription is regulated by ACTH, by gonadotropins in testis and ovary and by unknown factcors in placenta all activated through cAMP as intracellular second messenger (220). Each catalytic cycle requires a molecule of NADPH and one molecule of oxygen:

$$\text{cholesterol} + 3\text{NADPH} + 3H^+ + 3O_2 \xrightarrow{\textbf{P450}_{\textbf{SCC}}} \text{pregnenolone} + \text{isocapraldehyde} + 4H_2O + 3\text{NADP}^+$$

8.5.3 $P450_{C17}$

$P450_{C17}$ is the steroidogenesis qualitative regulator. It presents both 17-α-hydroxylase and C-17, 20-lyasic activities and represents a strategic point in the synthesis of steroid hormones as it can direct pregnenolone toward mineralocorticoids, glucocorticoids or sex steroids synthesis. Pregnenolone and progesterone can form respectively, 17-α-hidroxy-pregnenolon (17-OH-Preg) and 17-α-hydroxyprogesterone (17-OH-Prog) after 17-α-hydroxylation.

These 17-hydroxylated steroids then can be cleaved to give C17/20 DHEA and androstenedione, respectively. When the $P450_{C17}$ is absent, as in the zona glomerulosa, the products are C-21 17-deoxy steroids such as aldosterone. When the activity of 17-α-hydroxylase is present products are C-21 17-hydroxysteroids such as cortisol. Instead, when there are 17-α-hydroxylase and 17, 20 $P450_{C17}$ liasica activities the products are C-19 precursors of sex steroid hormones.

8.5.4 $P450_{C21}$

Progesterone and 17-OH-Prog, once synthesized, are hydroxylated in position 21 to give rise respectively to DOC (deoxycorticosterone) and 11-deoxycortisol. The P450 reductase $P450_{C21}$ uses the same used by $P450_{C17}$ for the transport of electrons from NADPH and is encoded by the gene called CYP21.

8.5.5 $P450_{C11}$ - $P450_{C18}$

P450C11 and P450C18 are located in the inner mitochondrial membrane. The human genome has two P450 genes located on chromosome 8 between bands q13 and q22 (221). These two genes encode P450 proteins that have 93% amino acid sequence identity.

P450C11 is encoded by the gene CYP11B1; it is significantly expressed in the fasciculata zone and is the only with 11-β-hydroxylase activity.

The related gene, the P450C18, is encoded by CYP11B2 and expressed, at very low level, only in the zona glomerulosa. The P450C18 has 11-β-hydroxylase, 18-hydroxylase and 18-oxidase activities (222). The gene CYP11B1, which encodes for P450C11, required for the synthesis of cortisol, is regulated by ACTH, whereas CYP11B2 gene, which encodes the P450C18 required for the synthesis of aldosterone, is regulated by angiotensin II (Ang II), sodium and potassium.

8.5.6 3βHSD

Once formed, pregnenolone can be converted into 17- idrossipregnenolone by P450C17 or in progesterone by 3-β-hydroxysteroid dehydrogenase Δ4-5Δ isomerase, encoded by the HSD3B gene.

This enzyme presents two activities: 3-β-hydroxysteroid dehydrogenase and isomerase activities.

In humans there are at least two forms of HSD3B, encoded by different genes:
- the gene for HSD3B type I (HSD3B1) is expressed in placenta, skin, mammary gland;
- the gene for HSD3B type II (HSD3B2) is expressed in adrenal glands and gonads.

Both genes are on band p13 of chromosome 1 (223).

8.5.7 β-steroid-sulfotransferase-sulfatase

The steroid sulfates can be synthesized directly from cholesterol sulfate or may be formed by sulfation of steroids such as DHEA, by means of a cytosolic sulphate transferase leading to DHEA-S, encoded by the gene SULT2A1.

The steroid sulfates can also be converted by hydrolysis in native form using a steroid-sulfatase.

8.5.8 17-chetosteroid-reductase

In adrenal, DHEA can be converted in Δ5-androstenediol and Δ4-androsterone in testosterone through 17-chetosteroid-reductase (17-CHSR) activity.

Δ5-androstenediol, testosterone and estradiol can also be converted respectively in DHEA, Δ4-androstenedione and estrone by the same enzyme, tank to a reversible activity known as 17-β- hydroxysteroid dehydrogenase (17-β-HSD). So this enzyme presents both androgenic and estrogenic 17-CHSR characteristics.

9. Adrenocortical cancers

9.1 Introduction

Adrenocortical cancers are rare tumor. Variuos treatment strategies have been valuated in several studies including considerable numbers of patients. Thus, therapeutic concepts based on solid experimental and clinical evidence are emerging.

Treatment is performed with curative intention in localized tumor stages. In advanced disease, highly individualized treatment goals include surgical mass reduction, control of endocrine activity and alleviation of symptoms from local tumor growth.

The range of therapeutic tools to target specific goals has expanded. Classic pharmacological tratment modalities and antineoplastic chemotherapy have been complemented by more experimental endocrinological and immunological interventions. Radiological strategies include local irradiation and systemic application of specific radioisotope-coupled agents.

Surgical techniques have recently been expanded by minimal invasive techniqes. Tumors that originate from the adrenal cortex can be divided into benign adenomas and malignant adenocarcinomas. They differ from other cancers because the cancer may be associated to an endocrine component (224).

Secreting forms are responsible for the onset of endocrine syndromes which vary depending on the type of hormone produced in excess:

- Cushing's syndrome, caused by hypersecretion of cortisol;
- Conn's syndrome, caused by aldosterone hypersecretion;
- hirsutism and virilization, caused by hypersecretion of androgens.

9.2 Epidemiology

Only indirect estimates of incidence and prevalence are available for adrenal carcinoma and malignant pheocromocytoma and they are in the range of three-four new cases per million inhabitants (225); according to older data (National cancer Institute 1975, (226), the incidence was estimeted to be between 0.5 and 2 per million inhabitants (227). However increasingly sensitive imaging techniques have ed to a more frequent detection of adrenal incidentalomas, and among them, some previously unsuspected adrenocortical carcinomas. ACCs are more frequent in women than in men, especially in those exposed to

estro-progestin (187). The age of distribution is bimodal, with a peak in the first decade and another in the fifth (228). Prognosis in malignant adrenal tumors is poor: the overall suvival rate is about 36% at five years and median survival is 28 months (229).

9.3 Pathogenesis

Adrenocortical carcinomas are monoclonal lesions. Among somatic mutation that can contribute to the malignant phenotype: overexpression of insuline-like growth factor II (IGFII) (185, 230), transforming growth factor α and epidermal growth factor (231). IGF-II gene shares its chromosomial locus (11p15) with two tumor suppressor gene p57(kip2) and H19 (232, 233); loss of heterozygosity or pathological imprinting has been demonstrated for this region and leads to IGF-II overexpression and loss of tumor suppressor gene p57(kip2), which encodes for a cyclin-dependent kinase, that leads to enhanced activity of G1 cyclin/cyclin-dpendent kinase complexes, which in turn promotes cell proliferation (232, 234).

In adrenocortical carcinoma p53 expression is higher than in adenoma (235) and mutations in the p53 gene were found more frequently in malignant tumors (236). Additionally, germ line mutations of p53 predispse to childhood adrenocortical cancer (237) (238).

The β-catenin, a key component of the Wnt pathway active during embryonic development, plays a structural role in cell-cell adherence. in both adrenocortical benign and malignant tumors can be observed an accumulation of β-catenin. These changes seem to be very frequent in ACCs an are in agreemet with the abnormal activation of the Wnt pathway in this type of tumor. This is explained by somatic mutations of β-catenin gene that alters the site of phosphorylation of glycogen synthase kinase 3-β (GSK3-β) (239). The GSK3-β is involved in β-catenin regulation of. In absence of Wnt signaling pathway, β-catenin level is low, because GSK3-β phosphorylates β-catenin at the critical amino acid residues in the N-terminal region, and the protein is degraded by the ubiquitin-proteasome. The stimulation of Wnt leads to inactivation of GSK3-β and then to the stabilization of β-catenin in the cytoplasm.

A mutation that leads to inactivation of the MEN1 germline is found in approximately 90% of families with multiple endocrine neoplasia type 1 (MEN1). Adrenocortical tumors and / or hyperplasia are observed in 25-40% of patients with MEN1 (240), (241). In most cases these are non-functional adrenocortical adenomas. Hyperplasia was found in a typical way in patients with MEN1 who have hypersecretion of ACTH (Cushing's syndrome), while

the ACC has been reported rarely in patients with MEN1. The mutation of the MEN1 gene in somatic cells is very rare in adrenocortical tumors (241), (242)).

9.4 Adrenocortical adenoma

It is a benign neoplastic proliferation of adrenocortical cells almost always associated with clinical, histological and instrumental evidences of hyperfunction.

Dimensions are variable depending on the hormone produced:

- adenoma with hyperaldosteronism is usually unilateral and of yellowish color, around 1.5 cm of size and non-enveloped;
- adenoma with hypercortisolism is unilateral, has dimensions of about 4 cm, is yellow-brown and is encapsulated;
- adenoma with virilization is unilateral, has dimensions of about 5 cm, is red-brown and is encapsulated.

In many patients, adrenal adenomas can't cause symptoms, if it doesn't produce an excess of hormones and is not large.

In case of large tumors, patients may have symptoms due to the compression of other organs, such as feeling of abdominal fullness or localized abdominal pain.

More frequent with advancing age, adrenocortical adenomas have a peak between 50 and 70 years and the most affected are women (58%) and the right side.

9.5 Adrenocortical carcinoma

About half of adrenal carcinomas are hyperfunctioning with excessive production of corticosteroids, mineralocorticoids and sex hormones.

The secretory forms are more frequent in children and young people, while in elderly patients, non-secretory form is more common and therefore diagnosed at an advanced stage for the clinical manifestations related to local growth or distant metastases.

In recent years, the spread of instrumental investigations led to occasional highlighting of non-symptomatic adrenal masses (incidentalomas) with relatively early diagnosis of not secreting malignancies.

Histologically standpoint are detectable: solid or trabecular areas with fibrous bands interposed between the tumor nodules, necrosis, the presence of large cells with vacuolated

cytoplasm, nucleus atypical and hyperchromatic, prominent nucleoli, frequent mitosis, evidence of vascular and capsular invasion.

It is highly aggressive: about 60% of patients have metastases at diagnosis, with a 5-year survival rate of 8% for recurrent and inoperable disease.

Cushing's syndrome is most frequently associated with endocrine cancer.

The therapeutic approach of choice for adrenocortical carcinoma is surgery.

Surgery should always be made, where possible, both in the treatment of primary cancer or in the event of local or distant recurrence.

Treatment is suitable for advanced or inoperable forms: mitotane is the drug most commonly used in the treatment of adrenal gland cancer, being reported partial and complete responses in 35% of patients treated.

However, the results of the treatment of advanced forms of carcinoma with mitotane are conflicting: some reports attest durable and complete remissions, while others attribute to mitotane a modest antineoplastic activity.

Treatments with cisplatin and etoposide in combination with mitotane are placed among the most active for in advanced cancer.

Other cytotoxic agents were used in the treatment of this disease such as vincristine, 5-fluorouracil and streptozotocin giving variable results (243, 244).

9.6 E2 and adrenocortical carcinoma proliferation

Adrenocortical cancer proliferation, correlated with p53 and ras gene family alterations and with up-regulation of IGF-II system, has recently been made in relation to exposure to physiological concentrations of E2.

A possible involvement of estrogen in tumor development was been suggested by epidemiological evidence and experimental studies: adrenal tumors, especially those secreting, are more frequent in women and the use of estrogen-progestin is a risk factor for tumor development.

H295R cells, able of converting androgens into estrogens by a constitutive expression of aromatase, expressing ERβ in larger amounts than Erα, are sensitive to low doses of estrogen in terms of proliferation (190).

H295R proliferation seems to be supported by the presence of an autocrine mechanism mediated by E2 through its receptors.

E2 involvement H295R cell proliferation was further proved by the assessment of response to estrogen receptor antagonists such as ICI 182 780 and OHT (4-OH tamoxifen).
This showed a dose-dependent inhibition of basal and E2-dependent cell proliferation. In particular, OHT induced morphological changes characteristic of apoptosis up-regulating the expression of FasL and inducing autocrine activation of caspases while ICI caused a cytostatic effect that could be explained by the inhibitory effects exerted by ICI on IGF signaling pathway, which is strongly activated in H295R by autocrine IGF-II action through the IGFIR.
ICI mediated inhibition of cell growth is therefore not solely attributable to competition between estrogen and ICI for the estrogen receptor but also to the interruption of the IGF signaling pathway (190).
In a recent study, always to test the role of estrogen in adrenal cancer onset, was investigated the expression profile of genes involved in sex hormones production.
ERα, ERβ, androgen receptor and aromatase are expressed by adrenal cortex and adrenocortical tumors. ERβ is the receptorial predominant type and it is constantly expressed in glomerular and fascicular zones. Western blot analysis revealed the existence of a truncated form of the androgen receptor in adrenocortical tissues. Levels of ERβ significantly lower, ERα up-regulation and aromatase over-expression are characteristic of the tumoral condition. In addition, the expression of ER was correlated with the expression of nuclear hormone receptors, suggesting that they may be involved in the modulation of ER. Results of this study suggest that estrogen produced locally by aromatase can induce the proliferation of adrenocortical cells through autocrine and paracrine mechanisms and open new perspectives on the potential use of anti-estrogens and aromatase inhibitors as therapeutic agents against adrenocortical carcinoma (189).

10. Aromatase expression in the adrenal gland under physiological and pathological condictions

Several studies conducted on normal adrenal gland regarding aromatase expression led to conflicting results.

Some studies revealed the absence of the enzyme expression in normal adrenal gland (245) while others detected the presence of a basal expression (246).

The first studies to assess the microsomal enzyme activities were conducted on normal human adrenal glands, adrenal tumor and placenta (used as a positive control): in this case no aromatase activity was detected in normal adrenal glands while very high aromatase activity was detected in tumor tissues (247).

Subsequently, studies on the fetal adrenal gland (248), on glomerulosa and medulla areas of normal adrenal gland (249), on human feminizing adreno-cortical tumors (250) and on NCI-H295R cells (190), have shown the presence of aromatase.

Western blot analysis allowed the visualization of the protein, as a double band of 55 kDa and 60, with confirmation of its expression in adreno-cortical normal tissues, at levels lower than those of the placenta (189). The small change in molecular weight could be due to a process of glycosylation, which however does not seem to affect enzyme activity ((251, 252).

Increased aromatase expression and CYP19 mRNA was observed in adreno-cortical carcinomas, especially in those secreting cortisol and androgens (189).

In adrenocortical carcinoma cells estrogens, synthesized by aromatase, herein over-expressed, mediate their proliferative effects through ERα, while the antiproliferative activity, exercised through ERβ, seems to be lost in tumor cells.

11. IGF-II/IGFIR pathway role in ACCs

The most consistent and dominant genetic change in adrenocortical carcinoma (ACC) is the perturbation of the insulin-like growth factor II (IGF-II) locus (11p15) that is imprinted. IGF-II is over-expressed in 90% of ACCs together with IGF1R. The direct involvement of IGF-II/IGF1R system in adrenocortical tumor cell proliferation has also been shown in vitro using adrenal cancer cell line H295R (185). It has been shown in several tissues that upon ligand binding, the intrinsic tyrosine kinase of the IGF1R caused the activation of phosphatidylinositol 3-kinase (PI3K)/AKT (253) and Raf-1/MEK/ERK pathways regulating positively cellular proliferation. In addition, receptors for growth factors like IGF-II are able to activate also protein kinase C (PKC).

Transgenic mice over-expressing IGF-II postnatally were generated and were demonstrated to have adrenocortical hyperplasia, although frank malignancy was not observed (254). This observation suggests that IGF-II is important for the abnormal proliferation of adrenal cells, but that additional steps are required for transformation to neoplasia. In addition, anti-IGF1R monoclonal antibody figitimumab has been used in phase-I clinical trial for the treatment of refractory adrenocortical carcinoma, however no objective responses were seen in the refractory ACC patients (255).

12. The nuclear receptor coregulator PELP1

12.1 Introduction

Despite the achievement of improvements in the treatment of estrogen dependent tumors (256, 257), initial or acquired resistance to endocrinee therapies frequently occurs. Emerging evidence suggests that ER action is complex, involves genomic as well as nongenomic signaling events (142, 144), and requires functional interactions with coregulators (192). As a modulator of ER functions, ER-coregulators are likely to play a role in cancer progression and emerging data suggest that the level and activity of coregulators can specify hormonal sensitivity of tumors. Advances in research during the past decade have identified several novel proteins as being ER coregulators (258). One of the ER coregulators, proline-, glutamic acid-, leucine-rich protein-1 (PELP1) (193), (also previously known as modulator of the nongenomic actions of the estrogen receptor, MNAR) (194), is unique because it plays important roles in both genomic (195) and nongenomic actions of the ER (196), (197).

Researchers first identified PELP1, as one of the several proteins that bind to the SH2 domain of Lck tyrosine protein kinase utilizing GST-SH2 pull down assays. In the SDS-Page gel this protein migrated as a 160-kDa protein, hence it was initially named p160 (not to be confused with the SRC/p160 family) (Figure 12.1). Functional studies revealed that in addition to Lck, PELP1 also interacts with other members of the Src kinase family, including c-Src (259). Subsequent studies identified orthologs to PELP1 in mice and rats (260). Homology data established that PELP is expressed in other mammals, including chimpanzees, dogs and cats.

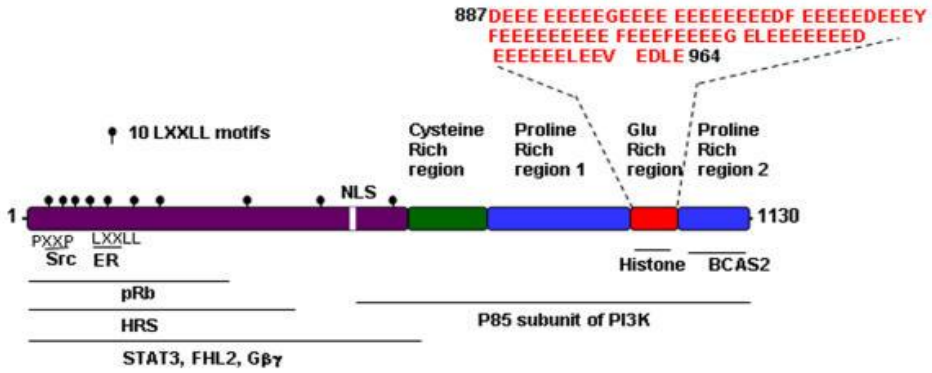


Figure 12.1. Schematic diagram of functional domains identified in PELP1/MNAR. PXXP, SH3 binding domain; LXXLL, nuclear receptor interacting domain; glu-rich, histone binding domain. Putative regions of interaction with other proteins are shown.

12.2 Protein domain structure

PELP1/MNAR contains several motifs and domains that are commonly present in many transcriptional coactivators, including 10 nuclear receptor (NR)-interacting boxes (LXXLL motifs), a zinc finger, a glutamic acid-rich domain, and 2 proline-rich domains (Figure 12.1). A unique feature of PELP1 is the presence of an unusual stretch of 70 acidic amino acids in the C-terminus that functions as a histone-binding region (195, 261). Interestingly, proline-rich regions contain several consensus PXXP motifs that may interact with signaling proteins containing SH3 domains. PELP1 encodes a protein of 1130 amino acids and has a predicted molecular weight of 120 kDa with an isoelectric point of 4.30, but because of its overall negative charge and excessive number of prolines, the protein migrates on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels as a 160-kDa protein.

12.3 Interactions with NRs

PELP1 appears to function as a general coregulator, as it contains 10 NR-interacting boxes (LXXLL) and interacts with multiple NRs. Estrogen promotes PELP1 interaction with the AF2 domain of ERα (193). LXXLL motifs 4 and 5 primarily mediate binding of PELP1 to ERα (196). PELP1 also interacts with ERβ (259). Using receptor subtype-specific ligands, researchers showed that PELP1 acts as a coactivator for both ERα and ERβ (262). PELP1 also interacts with several other NRs, including androgen receptors, glucocorticoid receptors, and progesterone receptors, in a ligand-dependent manner (193), (259). PELP1 interacts with retinoid X receptor (RXR)α and enhances transactivation in response to 9-*cis*

retinoic acid (RA) (263). Furthermore, PELP1 functions as a corepressor of non-NR sequence-specific transcription factors, including activating protein 1, NFκB, and ternary complex factor/serum response factor (261). PELP1 also interacts with the transcription factor signal transducer and activator of transcription (STAT)3 and enhances growth factor-mediated STAT3 transactivation functions (207).

12.4 PELP1-interacting proteins

PELP1/ interacts with several components of chromatin-modifying complexes, including CRE-binding protein-binding protein (CBP), p300, metastasis-associated protein (MTA)1, and histone deacetylase 2 (193, 261), as well as with histones (195, 261). Recent studies showed that PELP1 interacts with Sumo-2 and forms a complex with the SET domain, bifurcated 1 and lysine-specific demethylase 1, which are a histone lysine methyltransferase and demethylase, respectively (264). PELP1 also interacts with the transcriptional activator four-and-a-half LIM-only protein 2 (205) and with several components of spliceosomes, including breast carcinoma amplified sequence 2 and splicing factor 3b, indicating that PELP1 may play a role in coupling transcriptional activation with splicing (205, 265). Also, PELP1/MNAR interacts with several key components that play a role in cell cycle progression, including CDK4, cyclin D1, and retinoblastoma protein (pRb) (193, 207, 259, 262, 266, 267) and with the cytosolic kinases c-Src and phosphatidylinositol-3 kinase (PI3K) (259, 268). Mitogenic signaling promotes the association of PELP1 with a number of signaling components, including epidermal growth factor (EGF) receptor (EGFR), HER2, STAT3, and hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) (269). The diversity of the PELP1 -interacting proteins (Figure 2) and the ability of PELP1 to interact with histones and histone-modifying components and transcriptional regulators suggest that it plays a role in chromatin modifications and couples NRs with different signaling components (Figure 12.2).

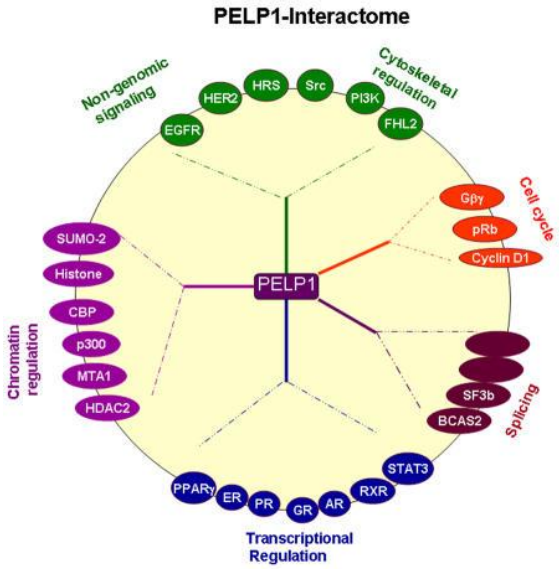


Figure 12.2. PELP1/MNAR interactome. Schematic representation of PELP1-interacting proteins, grouped on the basis of putative function.

12.5 Expression

PELP1 is expressed in a wide variety of tissues, with the highest levels of expression noted in the brain, testes, ovaries, and uterus (193, 260, 270, 271). In the mouse brain, PELP1 expression is developmentally regulated, and researchers have observed widespread PELP1 expression in the cortex, hypothalamus, and midbrain, as well as in neurons and astroglia (271). In the rat brain, a study reported high-intensity PELP1 staining in the known targets of estrogen/steroid actions, including the hippocampus, cortex, hypothalamus, amygdala, and septum (260).

PELP1 expression is developmentally regulated in mammary glands, with the highest levels of expression found during pregnancy (193). PELP1 expression is upregulated by estrogen signaling and differentially regulated by selective estrogen receptor modulators (272).

The PELP1 promoter region contains several consensus sites for retinoic acid receptor, and 9-*cis* RA transcriptionally upregulates the expression of the *PELP1* gene and PELP1 protein (263).

12.6 Subcellular distribution

PELP1 contains a central consensus nuclear localization site and exhibits both cytoplasmic and nuclear localization, depending on the tissue (193). PELP1 predominantly resides in the nuclear compartment of hormonally responsive tissues (193, 195). Immunohistochemical studies using a variety of mouse tissues revealed PELP1 staining in all of the tissues examined, but that PELP1 was localized differently in each organ (193). The protein was present in both the nuclei and cytoplasm to differing degrees, depending on the tissue. In ovaries and fallopian tubes in pregnant mice, PELP1 was localized predominantly in the nuclei of cells from the corpus luteum and the epithelium of fallopian tubes, but considerable cytoplasmic staining also occurred in these cells. In the lung, only parts of the epithelial cell nuclei were stained for PELP1, whereas in the testis, PELP1 was prominently localized in the nuclei. Using silver-enhanced nanogold and immunofluorescence staining of rat brain tissue, researchers showed that PELP1 was localized primarily in the nuclei of cells in various brain regions, with scattered staining in the cytoplasm and plasma membrane compartments (260). In endometrium, PELP1 exhibits distinct localization depending on the phase of the endometrium. In the proliferative and secretory phases, researchers showed that PELP1 was expressed in both the glandular and stromal compartments, and was localized in both the nucleus and cytoplasm of these cells (262). In contrast, in the postmenopausal phases, researchers observed PELP1 staining only in the glandular compartment, and that PELP1 expression was confined to the cytoplasm of these cells (262). Studies also reported PELP1 expression in all of the cell lines commonly used in laboratory research, including cancer cell lines; however, the level of expression varied among the cells (193, 270). In certain cancer cells, researchers reported the association of a small portion of PELP1 with the membrane (268, 270). Within the nucleus, PELP1 appears to be present in several subcompartments, including the chromatin, nucleoplasm, and nuclear matrix (195).

12.7 Post-translational modifications

PELP1 is a phosphoprotein and is phosphorylated on both serines/threonines and tyrosines. PELP1 contains several potential sites for phosphorylation, including 8 tyrosine kinase/phosphatase sites (recognized by EGFR, platelet-derived growth factor receptor, insulin receptor, Src, Jak2, and SHP1) and 207 serine/threonine kinase/phosphatase motifs (recognized by AKT, glycogen synthase kinase, CDK, casein kinase 1, casein kinase 2,

LKB1, mitogen-activated protein kinase (MAPK), protein kinase C, protein kinase A (PKA), and proline-directed kinases.

A recent study showed that PELP1 is phosphorylated by PKA and that PKA phosphorylates PELP1 at Ser-350, Ser-415, and Ser-613 (273, 274). Another study revealed that PELP1 is phosphorylated at Thr-745 in the developing brain (274).

PELP1 phosphorylation is modulated by hormones and growth factor signaling. Accordingly, treatment with EGF promotes tyrosine as well as serine phosphorylation of PELP1 (268). Estrogen treatment is shown to promote tyrosine phosphorylation of PELP1 at Tyr920 (199).

12.8 Enzyme activity

No studies have shown an association between PELP1 and known enzymatic activity. However, emerging evidence suggests that PELP1 functions as a scaffolding protein by coupling various signaling complexes with NRs. PELP1 associates with chromatin and interacts with histones (195). Although PELP1 itself has no histone acetyltransferase activity, it can recruit other coregulators with histone acetyltransferase activity, such as CBP and p300 (193). In addition, PELP1 interacts with components of histone deacetylase complexes, including the nucleosome remodeling and histone deacetylation complex protein MTA1 (275) and histone deacetylase 2 (261). The ability of PELP1 to interact with histones, histone acetyltransferase enzymes, and histone deacetylase enzymes suggests that PELP1 promotes alteration in local chromatin structure in the vicinity of the NR target promoters by coupling NRs with chromatin-modifying enzymes and by displacing histone H1 (195). A recent study also showed that PELP1 is associated with complexes that have a histone lysine methyltransferase and a histone lysine demethylase, suggesting that PELP1 has some function in these complexes (264). Furthermore, punctate subnuclear localization and nuclear matrix association of PELP1 suggest that PELP1 functions as a landing platform for several other chromatin remodeling complexes, thereby regulating gene expression.

Emerging evidence indicates that PELP1 also plays a key role in nongenomic ER activity. PELP1 modulates the interaction of ER with Src, stimulating Src enzymatic activity and MAPK pathway activation (259).

Mechanistically, PELP1 interacts with the SH3 domain of c-Src via its N-terminal PXXP motif, and ER interacts with the SH2 domain of Src at phosphotyrosine 537; the MNAR-

ER interaction further stabilizes this trimeric complex, leading to activation of the Ras/MAPK pathway (196). PELP1 also directly interacts with the p85 subunit of PI3K and enhances PI3K activity, leading to activation of the AKT pathway (268).

12.9 Target genes

PELP1 does not appear to have a DNA-binding domain. However, its ability to interact with several NRs suggests that it enhances the transcription of target genes via its interactions with NRs (193, 259). Accordingly, chromatin immunoprecipitation analysis has shown that PELP1 is recruited to the promoters of ERα target genes, including pS2, PR, and IGF (195). PELP1 regulates cyclin D1 expression at the transcriptional level; such regulation may involve functional interactions between PELP1 and pRb (200). Estrogen stimulation leads to enhanced recruitment of PELP1 to the MTA3 promoter chromatin, implying a role for PELP1 in the regulation of MTA3 (272). Additionally, growth factor signals promote PELP1 interactions with STAT3; these interactions play an important role in growth factor-mediated activation of STAT3 target genes, including cyclin D1, fos, and jun (207). In response to 9-cis RA stimulation, PELP1 enhances the expression of CRBP II via its interaction with RXR (263).

12.10 Chromatin remodeling and transcriptional activation

PELP1 exhibits a predominantly nuclear localization in many normal tissues. Within the nuclear compartment, PELP1 localizes to the nucleoplasm, chromatin, and nuclear matrix and is recruited to the promoters of several NR target genes (195). PELP1 co-localizes with acetylated histones and interacts with the acetyltransferases, CBP and p300, and the PELP1-associated histone acetyltransferase activity increases upon ligand-based treatment (195). Researchers have shown reduced histone H1 residence in target gene promoters during the time that PELP1 occupies these promoters (195). PELP1 is also associated with deacetylases, including components of nucleosome remodeling and the histone deacetylation complex (275), and inhibition of deacetylase activity increases the PELP1 residency time at the target gene promoter (195). Furthermore, a recent study reported that PELP1 exists as a complex with methyltransferases and methylases (264). PELP1 overexpression maintains NR-mediated nucleosomal alterations for an extended period (195). Collectively, these data suggest that PELP1 contributes to alteration of the local

chromatin structure required for the optimal transcriptional response by ligand-bound NRs via its interactions with histones and histone-modifying enzymes. In addition, PELP1 may play a role in NR-mediated splicing of mRNAs (265). PELP1 interacts with several components of the spliceosome machinery, including breast carcinoma amplified sequence 2 and splicing factor 3b, and co-localizes with the splicing factor SC35 in nuclear speckles. PELP1 has an RNA-binding domain, interacts with RNA in *in vitro* assays, and enhances steroid hormone-mediated splicing in minigene assays (265). These emerging data suggest that PELP1 mediates NR-mediated RNA splicing in addition to transcriptional activation functions.

12.11 Nongenomic signaling

Evolving evidence suggests that in addition to well-studied nuclear functions, the ERα also participates in nongenotropic (cytoplasmic and perhaps, membrane-mediated) signaling via formation of a multiprotein complex involving ERα, Src kinase, PELP1, PI3K, SHC, and G-proteins, collectively called the "signalsome" (145). Substantial evidence suggests that PELP1 participates in cytoplasmic and membrane-mediated signaling events through stimulation of the Src kinase, MAPK, PI3K, and STAT3 (146). Emerging evidence suggests that PELP1 is one of the coregulators that facilitate NR interactions with cytoplasmic signaling components (262, 269). Researchers initially identified PELP1 as a Src SH2-binding protein (276), and some portion of PELP1 exists in the cytoplasm and at the membranes (195, 262). PELP1 modulates ER's interaction with c-Src, stimulating c-Src enzymatic activity and leading to activation of the mitogen activated protein kinase (MAPK) pathway (259). Mutational analysis of ERα and c-Src mutants revealed that PELP1 interacts with the c-Src SH3 domain via its N-terminal PXXP motif. ERα interacts with Src's SH2 domain at phosphotyrosine 537, and the PELP-ER interaction further stabilizes this complex . In addition, PELP1 also directly interacts with the p85 subunit of PI3K and enhances PI3K activity (268). A recent study reported direct correlation between PELP1 expression levels and E2-induced activation of PI3 and Akt kinases. In their model system, E2 treatment induced complex formation of endogenous PELP1, ERα, c-Src, and p85, the regulatory subunit of PI3 kinase. The interaction between p85 and PELP1 required activation of c-Src and PELP1 phosphorylation on Tyr920. These results suggest that PELP1 plays a key role in the E2-induced regulation of cell proliferation and apoptosis via its regulation of the PI3K-AKT pathway (199). EGF promotes PELP1 association with the

EGFR, resulting in the tyrosine phosphorylation of PELP1 (268). PELP1 can enhance EGF-mediated ERα transactivation, and mislocalization of PELP1 in the cytoplasm can increase ERα basal activity via the EGFR and PI3K pathways. Using a yeast two-hybrid screen, (277) demonstrated the physiological interaction between HRS and PELP1/MNAR. Interestingly, HRS sequesters PELP1 in the cytoplasm, leading to EGFR-dependent activation of MAPK. PELP1 can interact with several growth factor signaling components, including STAT3, and may have important functional implications in ERα/growth factor crosstalk (268).

12.12 Cell cycle progression

Evidence suggests that PELP1 contributes to E2-mediated G1/S-phase progression (200). PELP1/MNAR interacts with pRb via its C-terminal pocket domain, and PELP1/pRb interactions play a role in the maximal activation of E2 target genes such as cyclin D1. Ratna et al (278) suggested that PELP1/MNAR positively contributes to E2-mediated G1/S-phase progression and plays a permissive role in E2-mediated cell cycle progression, presumably via its regulatory interactions with the pRb pathway. Increased PELP1 expression in a mammary gland during pregnancy, when the rate of cell proliferation is high, supports a physiological role for PELP1/MNAR in E2-mediated cell cycle progression in mammary glands (193). In addition to mitosis, emerging evidence indicates that PELP1 plays a role in meiosis. For example, in *Xenopus* oocytes, PELP1 interacts with androgen receptor (AR) and appears to mediate inhibition of meiosis via Gβγ signaling. PELP1 is widely expressed in oocytes, and reduction of its expression by RNA interference markedly enhances testosterone-triggered maturation and activation of MAPK. Furthermore, PELP1 appears to participate in maintaining meiotic arrest by directly enhancing G-mediated inhibition of meiosis and androgen binding to AR, releasing this inhibition, which allows maturation to occur (267).

12.13 PELP1 deregulation in hormonal cancers

Several lines of evidence implicate PELP1 as a potential proto-oncogene and its expression is deregulated in a wide variety of hormone-driven tumors including breast (198, 201-203, 278), endometrial (198), ovarian (204) and prostate cancer (205). Overexpression of PELP1 in fibroblasts and epithelial model cells results in cellular transformation and PELP1 over expression in breast cancer model cells potentiates rapid tumor growth in

xenograft studies (203). PELP1 interacts with and modulates functions of several proto-oncogenes, including c-Src, STAT3, and EGFR (269). PELP1 has been reported to be widely expressed in breast cancer cells (193, 270) and is shown to be up regulated in a subset of breast tumors (193, 262). A very recent study identified PELP1 as a cell fate determination factor (DACH1)-binding protein. DACH1 functions as an endogenous inhibitor of ERα transcriptional activity. Using 2,200 breast tumor samples, Popov et al., found that DACH1 expression is lost during breast cancer progression (279). Using mechanistic studies, they also demonstrated that DACH1 and PELP1 colocalize in the nucleus in ~80% of cells and that the relative balance of DACH1 and PELP1 in breast cancer cells has implications in ER signaling as DACH1 loss can potentiate PELP1 coactivation functions of ERα (279). Salivary duct carcinoma is a high-grade neoplasm with morphology similar to that of mammary duct carcinoma. Interestingly, these salivary tumors express PELP1 and ER, and PELP1 signaling may play a role in salivary tumorigenesis (268). Grivas et al., reported recently that PELP1 expression was higher in epithelial cells of colon carcinomas than in normal mucosa and that PELP1 overexpression in epithelial cells was found to be an independent favorable prognostic factor (280). PELP1 expression in myofibroblasts from normal mucosa as well as in carcinomas suggests that deregulated expression of co-regulators in both epithelial cells and myofibroblasts may contribute to the initiation and progression of colorectal carcinoma (281). Marquez-Garban et al., reported deregulated PELP1 expression in lung tumors (282). These emerging findings suggest that PELP1 is a novel proto-oncogene and its deregulation can potentially contribute to oncogenesis in hormone-driven cancers (Figure 12.3).

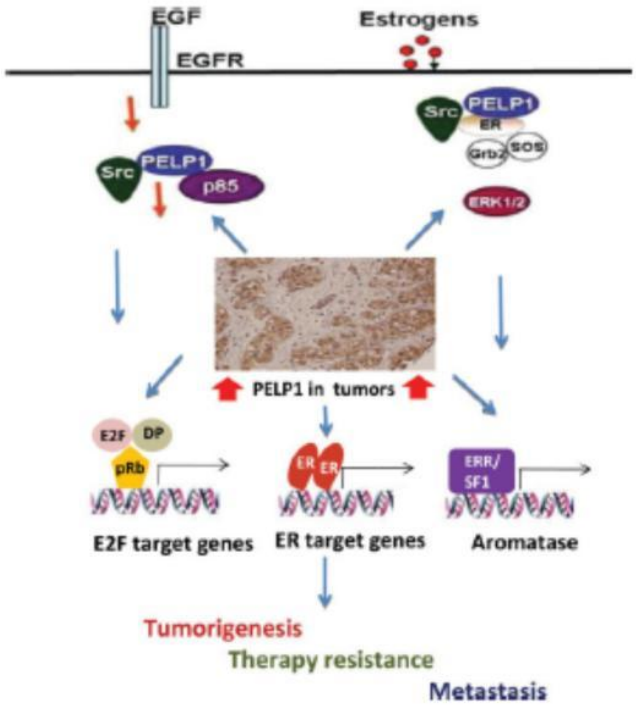


Figure 12.3. Schematic representation of the current understanding of PELP1 signaling pathway. PELP1 appears to function as a scaffolding protein that facilitates formation of various signalling complexes and its expression is deregulated in hormonal cancers. PELP1 regulates multiple signaling pathways including ER-genomic signaling by facilitating epigenetic changes, ERnongenomic signaling by activating Src-MAPK pathway, local estrogen synthesis via ERRα - aromatase signaling and cell cycle progression via pRb/E2F pathway. PELP1 deregulation in hormonal tumors is likely to contribute to the development of tumorigenesis, metastasis and hormonal independence by activating multiple signaling pathways and by facilitating ER signaling cross talk. Insert in the middle shows PELP1 expression in advanced breast tumors as analyzed by IHC.

12.14 Role of PELP1 in metastasis

PELP1 interacts with several proteins involved in cytoskeleton remodeling, including Src kinase, PI3K, and four-and-a-half LIM-only protein 2, and participates in E2-mediated nongenomic signaling pathways (259, 268, 283). Also, PELP1 overexpression uniquely enhances E2-mediated ruffles and filopodium-like structures. A recent study showed that in Boyden chamber assays, PELP1-overexpressing MCF-7 cells displayed increased cell motility upon treatment with E2, whereas knockdown of PELP1 expression by small interfering RNA substantially reduced E2-mediated cell motility compared with that in control MCF-7 cells (203). PELP1 modulates functions of MTA1, a protein implicated in

metastasis. PELP1 also interacts with the MTA1-associated coactivator and promotes ERα-transactivation functions in a synergistic manner (275). Additionally, PELP1 modulates expression of MTA3, a gene implicated in the invasive growth of human breast cancers. The ability of PELP1 to recruit to the MTA3 promoter chromatin (284) and its interactions with other MTA family members, suggest that deregulation of PELP1 expression promotes metastasis. A recent study measured the expression levels of PELP1 by IHC examination and the results showed that when compared with node-negative specimens, node-positive and metastatic tumors exhibited increased PELP1 expression (203). Statistical analysis revealed that PELP1 expression was positively correlated with cancer grade and node status. The number of samples with a high level (score 3) of PELP1 staining increased as tumors progressed from grade 1 to grade 2 or 3 ($P = 0.005$). Similarly, node-positive and metastatic tumors exhibited a greater expression of PELP1 than did node-negative tumors ($P = 0.003$). No significant correlation of PELP1 expression with ER, PR, patient age, or tumor stage was observed. These results suggest that PELP1 expression may be altered in higher grade node-positive and metastatic tumors (203).The ability of PELP1 to interact with various enzymes that modulate the cytoskeleton and its deregulation in metastatic breast tumors suggest that PELP1/MNAR signaling plays a role in tumor cell migration and metastasis.

13. Materials and Methods II

13.1 Cell culture and tissues

H295R cells (Figure 13.1) were obtained from Dr W.E. Rainey (Medical College of Georgia, Augusta, GA, USA) (285) and cultured in Dulbecco's modified Eagle's medium/Ham's F12 DMEM/F12 (Sigma) supplemented with 1% ITS Liquid Media Supplement (100X; Sigma), 10% fetal bovine serum, 1% glutamine, 2% penicillin/streptoycin and antibiotics (Sigma), at 37 °C in an atmosphere of humidified air containing 5% CO_2. Cell monolayers were subcultured onto 100 mm dishes for ChIP and IP assays (8 x 10^6 cells/plate), 60 mm dishes for protein and RNA extraction (4 x 10^6 cells/plate) and 24 well culture dishes for proliferation experiments (2 x 10^5 cells/well) and grown for 2 days in complete medium.

Prior to experiments, cells were starved overnight in DMEM/F-12 medium containing only antibiotics. Cells were treated with 17β-estradiol (E2) (100 nM) (Sigma), 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT) (1 μM) (Tocris Bioscience, Ellisville, Missouri, USA), IGF-II (100 ng/ml) (Sigma), AG1024 (AG) (10 μM) (Sigma), PD98059 (PD) (10 μM) (Calbiochem), LY294002 (LY) (10 μM) (Calbiochem), GF109203X (GFX) (10 μM) (Calbiochem) and IGF1R blocking antibody αIR3 (1 mg/ml) (Abcam, Cambridge, UK).

Fresh-frozen samples of adrenocortical tumors removed at surgery and normal adrenal cortex macroscopically dissected from adrenal glands of kidney donors were collected at the hospital-based Divisions of the University of Padua and Ancona (Italy). Tissue samples were obtained with the approval of local ethics committees and consent from patients, in accordance with the Declaration of Helsinki guidelines.

Diagnosis of malignancy was performed according to the histopathologic criteria proposed by Weiss et al. (286) and the modification proposed by Aubert et al. (287).

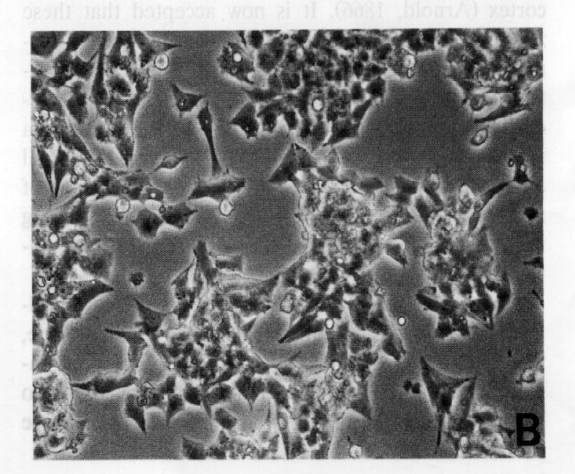


Fig.13.1 H295R cells

13.2 RNA extraction, reverse transcription and PCR

H295R cells were treated with E2 and PPT for 24 h. Total RNA was extracted with the TRizol RNA isolation system (Invitrogen). One microgram of RNA from each sample was used for RT-PCR with ImProm-II reverse transcriptase system kit (Promega). qPCR was performed using SYBR Green Universal PCR Master Mix (Bio-Rad, Hercules, CA, USA) using IGF1R specific primers:

Forward 5'-AAGGCTGTGACCCTCACCAT-3';

Reverse 5'-CGATGCTGAAAGAACGTCCAA-3'.

Relative gene expression was normalized to a calibrator that was chosen to be the basal condition (untreated sample). Results were calculated with the ΔΔCt method and expressed as n-fold differences in IGFIR gene expression relative to 18S rRNA and calibrator and were determined as follows:

$$\text{n-fold} = 2^{\Delta\Delta Ct}$$

$$(\Delta\Delta Ct = \Delta Ct \text{ sample} - \Delta Ct \text{ calibrator})$$

where the parameter Ct (threshold cycle) is defined as the fractional cycle number at which the PCR reporter signal passes a fixed threshold. ΔCt values of the sample and calibrator are determined by subtracting the average Ct value of the transcript under investigation from the average Ct value of the 18S rRNA gene, for each sample.

Human 18S rRNA primers were purchased from Applied Biosystems (Foster City, CA, USA).

13.3 Western blot analysis

Phosphorylation levels were determined 10 min after treatment, when inhibitors were used they were added 30 min before stimulus. Changes in expression levels were analysed 24 h after treatment. H295R cells and normal or cancer adrenal tessue samples 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. Fifty μg of protein were subjected to western blot analysis. Blots were incubated overnight at 4°C with specific antibodies: a) anti-pIGF1R antibody (Y1135) (DA7A8) (1:500; Cell Signaling Technology), b) anti-IGF1Rβ antibody (C-20) (1:800; Santa Cruz Biotechnology), c) anti-pERK1/2 antibody (T202/Y204) (1:500; Cell Signaling Technology), d) anti-ERK1/2 antibody (1:1000; Cell Signaling Technology), e) anti-pAKT1/2/3 (Ser473)-R (1:500; Santa Cruz Biotechnology), f) anti-AKT1/2/3 (H-136) (1:500; Santa Cruz Biotechnology), g) anti-human P450 aromatase antibody (1:200; Serotec), h) anti-SF-1 (1:10,000; provided by Prof. Ken-ichirou Morohashi National Institute for Basic Biology, Okazaki, Japan), i) anti-pCREB antibodies [Ser133] (1:1,000; Upstate Biotechnology, Temecula, CA, USA), j) anti-CCND1 antibody (3H2043) (1:1,000; Santa Cruz Biotechnology), k) anti-pERα (S118) (16J4) (1:500; Cell Signaling Technology), l) anti-pERα (S167) (D1A3) (1:500; Cell Signaling Technology), m) anti-ERα (F-10) antibody (1:1,000; Santa Cruz Biotechnology). Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham) and immunoreactive bands were visualized with the ECL western blotting detection system (Amersham). To assure equal loading of proteins membranes were stripped and incubated overnight with GAPDH antibody (GAPDH (FL-335) 1:2000; Santa Cruz Biotechnology).

13.4 Assessment of cell proliferation

3-[4,5-Dimethylthiaoly]-2,5-diphenyltetrazolium bromide (MTT) assay was conducted to detect cell proliferation. Cells were treated for 48 h in DMEM F-12 medium containing only 2% Pen/Strep. When IGF1R monoclonal antibody was used it was added 12 h before

treatment. Forty-eight h after treatment fresh MTT (Sigma) re-suspended in PBS was added to each well (final concentration 0.33 mg/ml). After 30 min incubation, cells were lysed with 1 ml of DMSO (Sigma). Each experiment was performed in triplicates and the optical density was measured at 570 nm in a spectrophotometer.

13.5 Chromatin immunoprecipitation assay

This assay was performed using the chromatin immunoprecipitation assay kit from Upstate (Upstate) with minor modifications in the protocol. H295R cells were grown in 100-mm plates. Confluent cultures (90%) were treated with E2 (100 nM) (Sigma), 100 ng/mL IGF-I (Sigma) alone or in combination with AG1024 (AG) (10 μM) (Sigma), PD98059 (PD) (10 μM) (Calbiochem), LY294002 (LY) (10 μM) (Calbiochem), GF109203X (GFX) (5 μM) (Calbiochem), or left untreated. Following treatment, DNA/protein complexes were cross-linked with 1% formaldehyde at 37°C for 15 min. Next, cells were collected and resuspended in 400 μL of SDS lysis buffer (Upstate) 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) 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), 1 μg of specific antibody anti-pCREB (Upstate) or 1 μg of specific antibody anti-ERα (F-10) antibody (1:1,000; Santa Cruz Biotechnology) 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, pCREB ad ERα, 5 μl volume of each sample and input were used for real time PCR using primers for the CYP19 promoter II:

forward, 5′-AACAGGAGCTATAGATGAAC-3′;

reverse, 5′-CAGAGATCCAGACTCGCAATG-3′;

primers for the CRE site in the IGF1R promoter:

forward, 5′-CTCGAGAGAGGCGGGAGAGC-3′;
reverse, 5′-GGAGCGGGGCCGAGGGTCTG-3′;
primers for the AP-1 site in the CCND1 promoter:
forward, 5′-GAGGGGACTAATATTTCCAGCAA-3′;
reverse, 5′-TAAAGGGATTTCAGCTTAGCA-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) 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 for the real time experiments, using input Ct values instead of the 18S, calibrator was basal sample.

13.6 Immunoprecipitation assay

H295R cells 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 and 500 µg of protein lysates were first precleared with 10 µl of protein A agarose beads (Calbiochem) for 1 h. Then cell lysates were incubated over night with primary antibody anti-PELP1 at 4°C in HNTG buffer (20mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.1 mM Na_3VO_4). In control samples, the primary Abs were substituted with nonimmune IgGs. The immunoprecipitated proteins were washed three times with HNTG buffer, separated on a 11% polyacrylamide denaturing gel, analysed by WB and visualized by ECL chemiluminescence (Amersham).

13.7 RNA interference

The ERα and PELP1 siRNA and non targeting siRNAs were purchased from Ambion (Applied Biosystems). Cells were plated into 60 mm dishes at $4x10^6$ cells, for protein extraction, and into 24-well paltes at $2x10^6$ cells for proliferation assay and used for transfection 48 h later. siRNAs were transfected to a final concentration of 50 nM using the

Lipofectamine 2000 and was used according to the manufacturer's recommendations (Invitrogen). Erα and PELP1-specific knockdown were checked by western analysis of proteins extracted from cells transfected for 48 h and treated for 24 h. Proliferation was evaluated for cells transfected for 24 h and treated for 48 h.

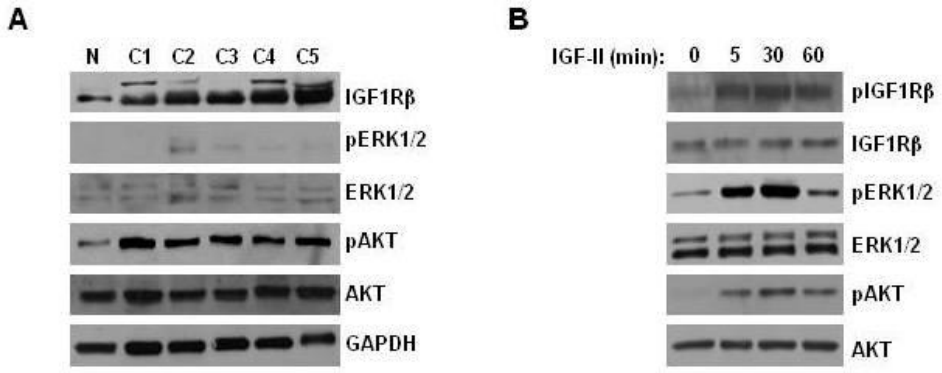
13.8 Data analysis and statistical methods.

All experiments were conducted at least three times and the results were from representative experiments. Data were expressed as mean values ± standard deviation (SD), and the statistical significance between control (basal) and treated samples was analysed with SPSS10.0 statistical software. The unpaired Student's *t* -test was used to compare two groups. $P < 0.05$ was considered statistically significant.

14. Results II

14.1 IGF-II signaling is active in human ACCs tissues and in H295R cells and is involved in cell proliferation.

Considering IGFIR up-regulation in human ACCs (183), we investigated the expression of genes related to IGF-II signaling in five different human ACCs samples. Western analysis revealed high expression levels of IGFIR and of the phosphorylated form of AKT (pAKT), compared to normal adrenal (Fig. 14.1 A), while we did not found a marked increase in the phosphorylated levels of ERK1/2 (pERK1/2) (Fig. 14.1 A). In order to determine if H295R cell line is a useful experimental model to study human ACC, we investigated in these cells IGF-II pathway and the effect of IGF pathway inhibitors on cell proliferation. Treating cells for increasing times with IGF-II we found a rapid increase in IGF1R, phosphorylation (Fig. 14.1 B). Similarly, ERK1/2 and AKT were rapidly activated by IGF-II, with maximum induction observed 30 min after treatment (Figure 14.1 B). Specificity of these activations was confirmed by the use of inhibitors, AG for IGF1R (Fig. 14.1 C), PD for ERK1/2 (Fig. 14.1 D), LY for PI3K/AKT (Fig. 14.1 E). AG blocked the activation produced by IGF-II on all kinases (Fig. 14.1 C-E). In addition, to confirm that IGF-II/IGF1R pathway influences cell cycle in ACC, we evaluated H295R cell proliferation in response to IGF-II used alone or in combination with AG, PD, LY and GFX, a specific PKC inhibitor (Fig. 14.1 F). IGF-II induced cell proliferation by 1.3 fold, while the presence of inhibitors blocked both basal and IGF-II-dependent cell proliferation (Fig. 14.1 F). The most effective inhibition was seen with AG that produced a 70% decrease in cell proliferation.



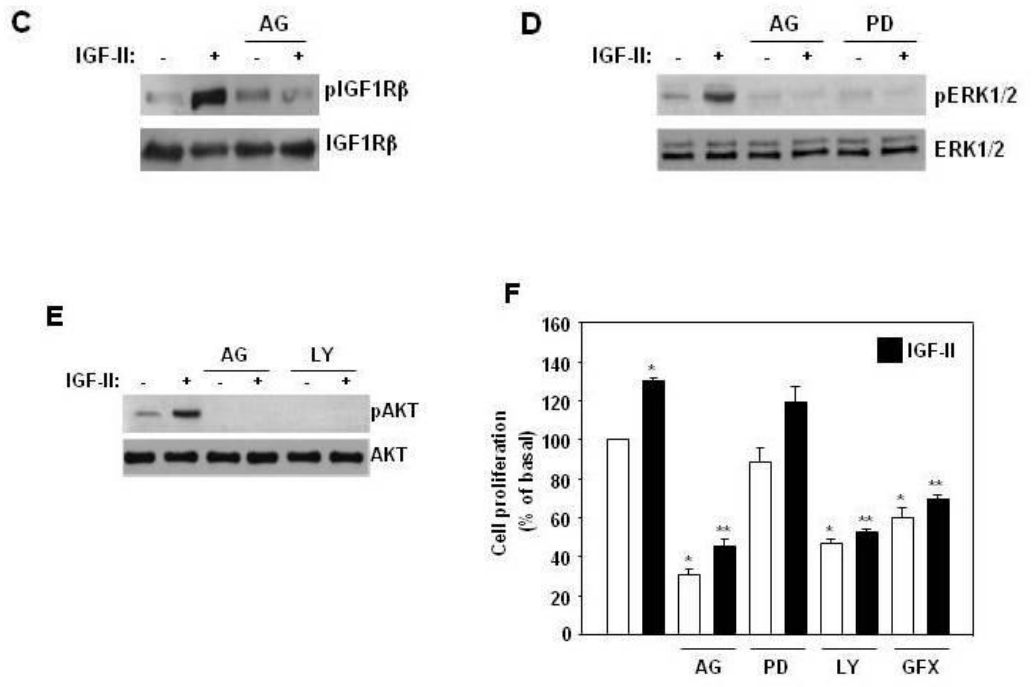
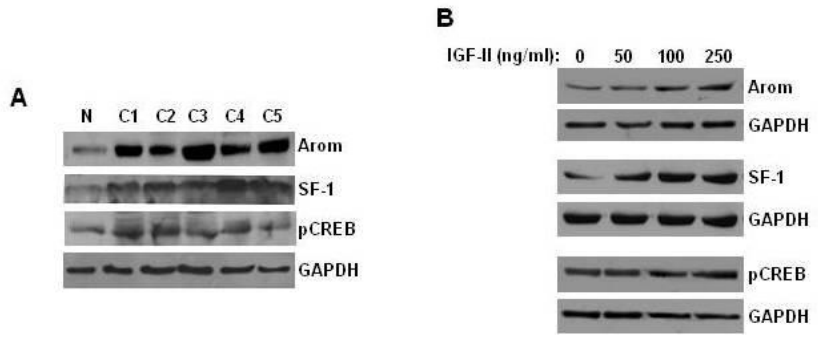


Figure 14.1. IGF-II signaling is active in human ACCs tissues and in H295R cells and is involved in cell proliferation. A, Immunoblot analyses for IGF1R, pAKT and pERK1/2 were performed on 50 μg of total proteins extracted from human normal adrenal tissues (N) and ACCs (C1-C5). B, Western blot analyses of pIGF1R, pERK1/2 and pAKT were performed on 50 μg of total protein extracted from H295R cells untreated or treated for the indicated times with IGF-II (100 ng/ml). GAPDH was used as a loading control. Results are representative of three different experiments. C, Immunoblot analysis for pIGF1R was performed on 50 μg of total protein extracted from H295R cells untreated or treated for 10 min with IGF-II (100 ng/ml) and AG1024 (10 μM) alone or in combination. IGF1R was used as a loading control. Results are representative of three different experiments. D, Immunoblot analysis for pERK1/2 was performed on 50 μg of total protein extracted from H295R cells untreated or treated for 10 min with IGF-II (100 ng/ml) and AG1024 (10 μM) and PD98059 (10 μM) alone or in combination. Total ERK1/2 was used as a loading control. Results are representative of three different experiments. E, Immunoblot analysis for pAKT was performed on 50 μg of total protein extracted from H295R cells untreated or treated for 10 min with IGF-II (100 ng/ml), AG1024 (10 μM) and LY294002 (10 μM) alone or in combination. Total AKT was used as a loading control. Results are representative of three different experiments. F, H295R cells were left untreated or treated for 48 h with AG1024 (10 μM), PD98059 (10 μM), LY294002 (10 μM), GF109203X (5μM), alone or in combination with IGF-II (100 ng/ml). H295R proliferation was evaluated by MTT assay. Results are representative of three independent experiments. Statistically significant differences are indicated (*, $P<0.05$ compared with basal; **, $P<0.01$, compared with IGF-II).

14.2 SF-1 and aromatase are highly expressed in human ACCs tissues and regulated by IGF-II/IGF1R pathway.

We have previously shown that ACCs are also characterized by aromatase up-regulation (189). We confirmed these data in our ACC samples and showed a marked increase in the levels of phosphorylated cAMP responsive element binding protein (pCREB) and steroidogenic factor-1 (SF-1) (Fig. 14.2 A), two transcription factors regulating aromatase expression through the PII promoter (191). In H295R cells aromatase is also highly expressed (190, 191). Here we found that IGF-II up-regulated aromatase expression in a dose dependent manner (Fig. 14.2 B). and that inhibitors for IGF-II pathway were able to decrease this induction (Fig. 14.2 C).

The effects on aromatase were dependent on increased expression of SF-1 (Fig. 14.2 B) while we did not found any change in pCREB levels (Fig. 14.2 B). Similarly to what observed on aromatase, IGF-II-induced SF-1 expression was decreased by AG, PD, LY and GFX (Fig. 14.2 C), while the same inhibitors did not affect pCREB levels (Fig. 14.2 C). To confirm the involvement of SF-1 in the control of aromatase expression we performed ChIP analysis that revealed increased binding of SF-1 to the human aromatase PII promoter after IGF-II treatment (Fig. 14.2 D), this binding was decreased by AG, LY and GFX but not PD. No changes were observed in pCREB binding levels (Fig. 14.2 E).



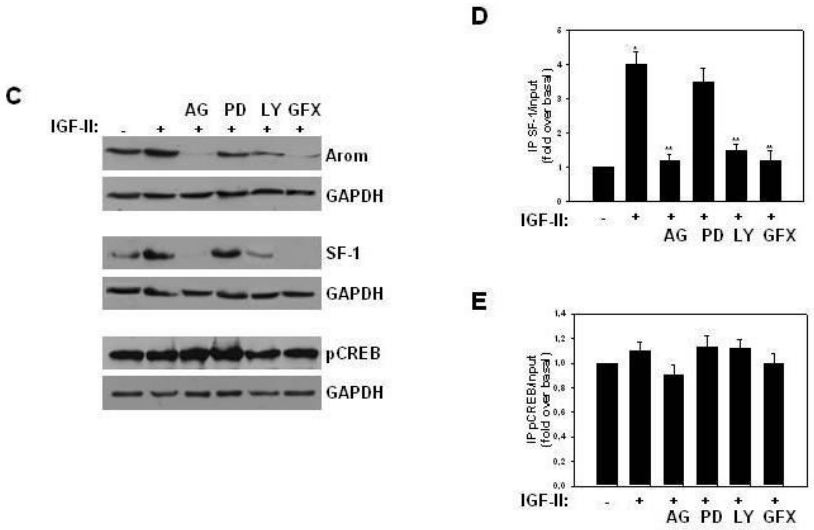


Figure 14.2. **SF-1 and aromatase are highly expressed in human ACCs tissues and regulated by IGF-II/IGF1R pathway.** A, Immunoblot analyses of aromatase (Arom), SF-1 and pCREB in human normal adrenal tissues [N] and ACCs [C1-C5]. (B) Immunoblot analyses of aromatase (Arom), SF-1 and pCREB (B) in H295R cells treated for 24 h with IGF-II at the indicated doses. (C) Immunoblot analyses of aromatase (Arom), SF-1 and pCREB in H295R cells treated for 24 h with AG, PD, LY, GFX in combination with IGF-II. GAPDH was used as a loading control. (D and E) H295R cells were treated with AG1024 (10 μM) and PD98059 (10 μM) LY294002 (10 μM), GF109203X (5μM), in combination with IGF-II (100 ng/ml). In vivo binding of SF-1 (D) or pCREB (E) to the aromatase PII promoter was examined using ChIP assay after 24 h treatment. Results are representative of three independent experiments. Statistically significant differences are indicated (*P<0.001 compared with basal; **P<0.001 compared with IGF-II).

14.3 Estrogens induce IGF1R expression through pCREB recruitment to IGF1R gene promoter.

Established a role for IGF-II in the control of aromatase expression and consequently on estrogen production, we wanted to evaluate if estrogens, through ERα, control ACC proliferation and IGF1R expression. A 48 h treatment of H295R with E2 and a ERα specific agonist, PPT, caused a significant increase in cell proliferation (Fig. 14.3 A). H295R cells were treated with E2 and PPT for 24 h, and IGF1R expression was evaluated at both mRNA and protein levels (Fig. 14.3 B and C). Results obtained demonstrated increase in IGF1R up-regulation in response to E2 and PPT (Fig. 14.3 B and C). IGF1R mRNA was up-regulated by E2 and PPT by 2 and 2.5-fold, respectively (Fig. 14.3 B). In an attempt to define how estrogens could induce IGF1R expression, we evaluated expression, in response to E2 and PPT, of pCREB, a transcription factor controlling IGF1R gene transcription (288), highly expressed in human ACC samples (Fig. 14.3 A). We found that in our cell line pCREB was strongly activated by E2 and PPT treatment (Fig. 14.3 D) and ChIP experiments clarified that E2 enhanced by 2-fold pCREB binding to IGF1R promoter (Fig. 14.3 E).

In addition, since minutes after administration E2 can activate IGF1R signaling in normal and malignant cells of various origins (139), we wanted to determine if this occurred also in ACC. We treated cells for increasing times with E2 (Fig. 14.3 F) and found that IGF1R, ERK1/2 and AKT were rapidly phosphorylated. To demonstrate that the effect was directly dependent on ERα, we also used PPT that was able to reproduce the effects seen with E2 (Fig. 14.3 G).

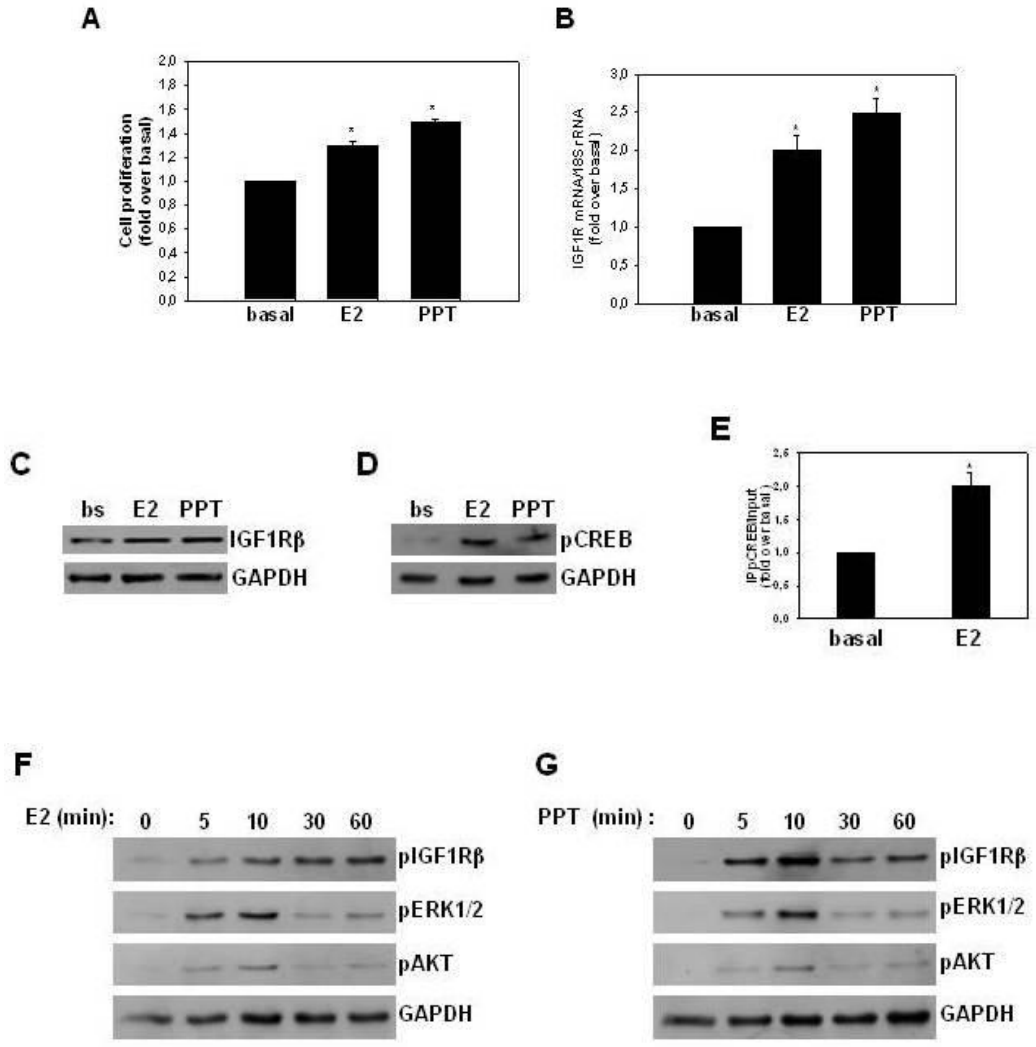
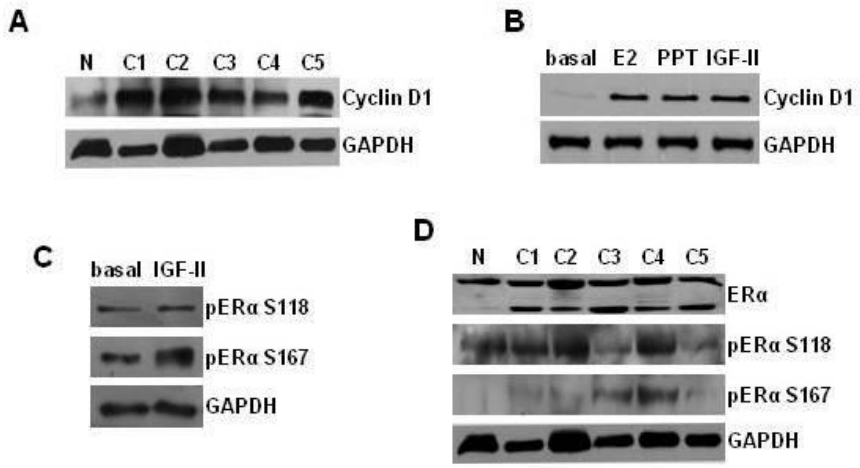


Figure 14.3. Estrogens induce IGF1R expression through pCREB recruitment to IGF1R gene promoter. A, H295R cells were left untreated (basal) or treated for 48 h with E2 (100 nM) and PPT (1 μM). H295R cell proliferation was evaluated by MTT assay. B, H295R cells were treated with E2 and PPT. Total RNA was extracted, and real-time RT-PCR was used to analyze IGF1R mRNA levels. C and D, Western blot analyses of IGF1R (C) and pCREB (D) were performed on 50 μg of total protein extract from H295R left untreated or treated for 24 h with E2 (100 nM) or PPT (1 μM). E, After chromatin immunoprecipitation, using an anti-pCREB antibody, total DNA was extracted and real time RT-PCR was used to analyze pCREB binding to IGF1R promoter after 24 h treatment. F and G, Immunoblot analysis for pIGF1R, pERK1/2 in H295R cells treated for the indicated times with E2 (F) and PPT (G). Results are representative of three independent experiments. Statistically significant differences are indicated (*P<0.01 compared with basal).

14.4 E2 and IGFII increase cyclinD1 expression via ERα.

Demonstrated the role for ERα in the control of IGF1R expression, we investigated if this receptor could have a role in the control of H295R cell proliferation altering expression levels of cyclin D1, a gene that is associated with both IGF/IGF1R transdutional pathway and ERα genomic activities (289). We first demonstrated an increase in cyclin D1 expression in human ACC tissues (Fig. 14.4 A).

Treatment with IGF-II, E2 and PPT caused an increase in cyclin D1 protein levels (Fig. 14.4 B). Stimulation of CCND1 mRNA transcription occurs through the interaction of ERα with several regulatory regions present in the promoter region such as binding motifs for AP-1 and Sp-1 (290). IGF-I is known to activate ERα transcriptional activity in a ligand-independent manner through phosphorylation on serine 118 and 167 (291, 292). To correlate the increase in cyclin D1 expression under IGF-II treatment with ERα expression we assessed whether IGF-II could increase ERα activation in H295R cells. Ten min treatment with IGF-II caused an increase in ERα phosphorylation on both serines (Fig. 14.4 C) and high phosphorylation levels were also found in ACC tissues where we confirmed higher ERα expression (Fig. 14.4 D). Furthermore, both IGF-II and E2 increased ERα binding to the AP-1 site of CCND1 promoter (Fig. 14.4 E).



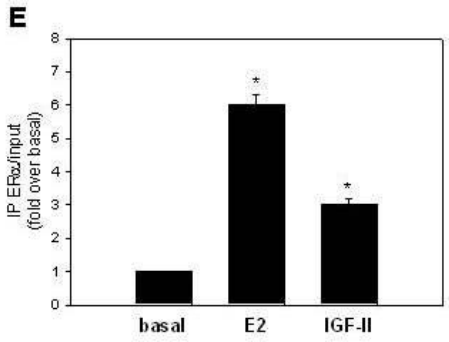


Figure 14.4. E2 and IGF-II increase ERα recruitment to CyclinD1 promoter. A and D, Immunoblot analyses for cyclin D1. A and B Immunoblot analysis for cyclin D1 in human normal adrenal tissues [N] and ACCs [C1-C5] (A) and in H295R cells treated for 24 h with E2 (100 nM), PPT (1 μM) and IGF-II (100 ng/ml) (B). Immunoblot analysis for phospho ERα serine 118 (pERα S118), phospho ERα serine 167 (pERα S167) in H295R treated with IGF-II (100 ng/ml) for 10 min (C). Immunoblot analysis for phospho ERα serine 118 (pERα S118), phospho ERα serine 167 (pERα S167) and ERα in human normal adrenal tissues [N] and ACCs [C1-C5] (D). GAPDH was used as a loading control. E, In vivo binding of ERα to the cyclin D1 promoter after 1 h treatment with E2 (100 nM) and IGF-II (100 ng/ml) was examined using ChIP assay. Results are representative of three independent experiments. Statistically significant differences are indicated (*P<0.001 compared with basal).

14.5 ERα silencing blocks E2 and IGF-II-dependent H295R cell proliferation.

We then wanted to compare the ability of an IGF1R monoclonal antibody and an ERα siRNA in controlling E2- and IGFII-dependent H295R cell proliferation. The presence of IGF1R blocking antibody αIR3 decreased IGF1R protein levels (Fig. 14.5 A) and had an effect on cell proliferation. Basal proliferation decreased by 50%, IGF-II induced a 1.4-fold increase in cell proliferation ad αIR3 decreased this effect by 50%. In the presence of E2 the inhibition was only 25% (Fig. 14.5 B).

A specific ERα siRNA allowed silencing of gene expression in H295R cells (Fig. 14.5 C) and in these conditions we observed a reduction in basal (70%), E2- (77%) and IGF-II-induced (72%) cell proliferation (Fig. 14.5 D).

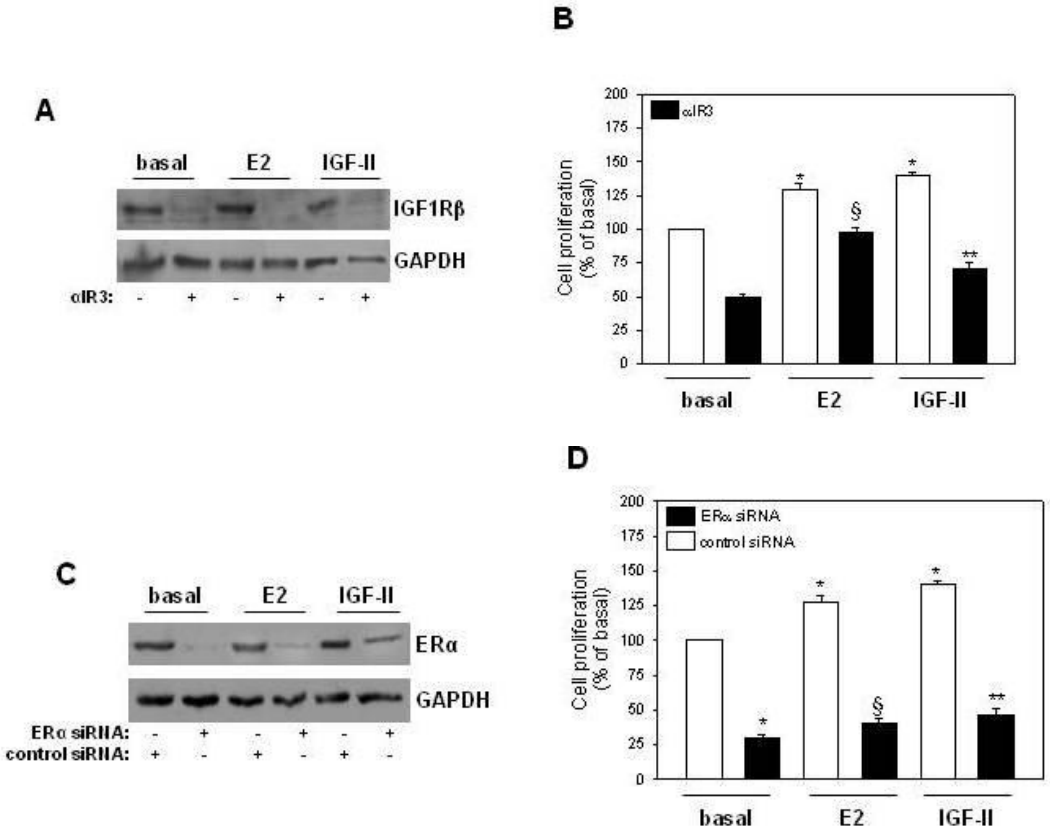


Figure 14.5. ERα silencing blocks E2 and IGF-II induced H295R cell proliferation. A and B, Where indicated H295R cells were incubated over night with αIR3 before being treated for an additional 24 (A) or 48 (B) h with IGF-II (100 ng/ml), E2 (100 nM) or vehicle (basal). C and D, H295R cells were transfected with ERα siRNA or a non targeting siRNA (control siRNA). Forty-eight (C) or 24 (D) hours after transfection cells were treated for an additional 24 h (C) or 48 h (D) with IGF-II (100 ng/ml), E2 (100 nM) or vehicle (basal). Immunoblot analyses of IGF1R (A) and ERα (C). B and D, Reduction of IGF-II and E2-induced H295R cell proliferation by αIR3 (B) siRNA and by ERα (D). Results are representative of three independent experiments. Statistically significant differences are indicated (*, P<0.0001 compared with basal; **, P<0.001 compared with IGF-II; §, P<0.01 compared with E2).

14.6 PELP1 is recruited to form a multiprotein complex in H295R after treatment with E2 and IGF-II.

In an attempt to define which was the scaffold protein involved in the cross-talk between estrogens and IGF-II pathways we focused our attention on Proline-, glutamic acid-, leucine-rich protein-1 (PELP1) (193), a scaffold protein of growing interest because it is deregulated in hormone-dependent carcinomas (293).

We treated H295R cells with E2 and IGF-II for 10 min and, after immunoprecipitation with anti-PELP1 antibody, showed the formation of a multiprotein complex formed by PELP1, IGFIR, ERα and Src (Fig. 14.6).

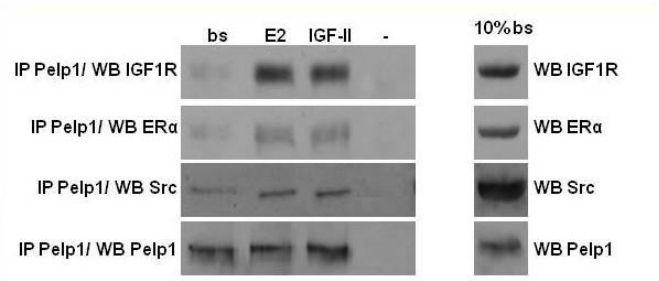


Figure 14.6. PELP1 is recruited to form a multiprotein complex in H295R after treatment with E2 and IGF-II. H295R cells were treated for 10 min with E2 (100 nM) and IGF-II (100 ng/ml). Total protein extract (500 μg) were immunoprecipitased with 1 μg of anti-PELP1 antibody. The samples were immunoblotted for IGFIR, ERα, c-Src. Protein expression for each sample was normalized to PELP1 content. Results are representative of three independent experiments. Statistically significant differences are indicated (*P<0.001 compared with basal).

14.7 PELP1 knock down decreases ERK1/2 phosphorilation in H295R.

Demonstrated the role of PELP1 as scaffold protein in the multiprotein complex formed after treatment with E2 and IGF-II between IGFIR, ERα and Src, we aimed to clarify the importance of PELP1 in rapid and long term action of E2 and IGF-II. We silenced PELP1 expression using a specific siRNA for 48 h and then treated H295R cells with E2 and IGF-II for 10 min. We observed that E2 and IGF-II loss their ability of inducing MAPK activation after PELP1 silencing (Fig. 14.7).

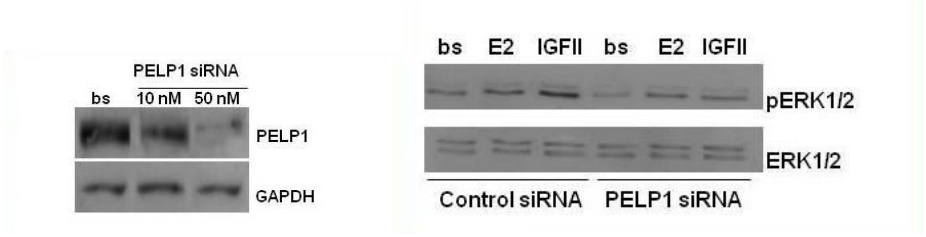


Figure 14.7. PELP1 knock down decreases ERK1/2 phosphorilation. H295R cells were transfected with PELP1 siRNA for 48h. After transfection cells were treated for 10 min with E2 and IGF-II. Western blot analyses of PELP1 and pERK1/2 were performed on 50 μg of total protein. Results are representative of three independent experiments. GAPDH and ERK1/2 were used as a loading control.

14.8 PELP1 knock down decreases IGFIR expression in H295R.

Starting from our previous observation that in H295R cells estrogens can induce IGFIR expression through a pathway involving ER/ERK/pCREB pathway (288) and considering that PELP1 silencing determines E2 inability to activate MAPK, we wanted to investigate if PELP1 silencing also determines a reduced expression of IGFIR. We silenced PELP1

expression using a specific siRNA and then treated H295R cells with E2 for 24 h and confirmed a reduced IGFIR expression (Figure 14.8).

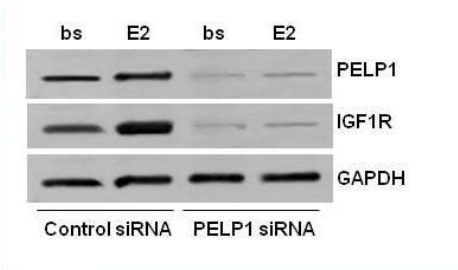


Figure 14.8. PELP1 knock down decreases IGFIR expression in H295R cells. H295R cells were transfected with PELP1 siRNA for 48h. After transfection cells were treated for 24h with E2 (100 nM). Western blot analyses of PELP1 and IGFIR were performed on 50 µg of total protein. Results are representative of three independent experiments. GAPDH was used as a loading control.

14.9 PELP1 knock down decreases Aromatase expression in H295R.

Considering that aromatase is overexpressed in ACC (189, 190) and that PELP1 acts as a cofactor able to induce aromatase transcription in breast cancer cells, we also evaluate PELP1 silencing on aromatase expression in H295R cells. We first silenced PELP1 expression and than treated H295R cell with IGF-II for 24h. Our experiments show that PELP1 silencing reduced aromatase expression levels both in basal condition or after stimulation with IGF-II (Fig. 14.9 A).

A previous study show that PELP1 regulates transcriptional activity of the aromatase gene by recruiting ERRα to a region located between promoter I.3 and II of the *aromatase* gene that controls aromatase expression in breast cancer cells (294). Considering that PELP1 through LXXLL region could interact also with SF-1 and activate aromatase transcription in ACC, we investigated if in H295R, PELP1 ad SF-1 could immunoprecipitate. We immunoprecipitate total protein lysates with PELP1 and showed an increase in SF-1 expression after treatment with IGF-II for 6 h (Fig. 14.9 B).

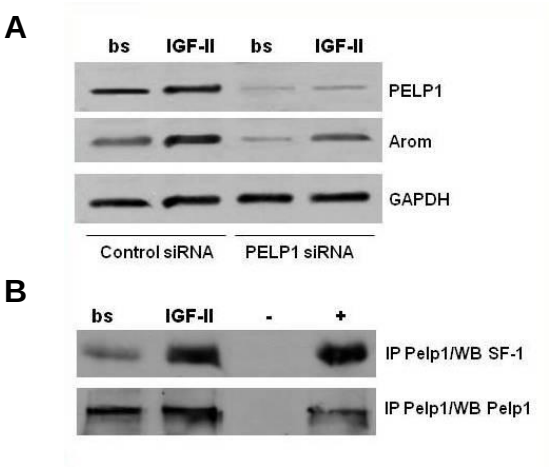


Figure 14.9. PELP1 knock down decreases Aromatase expression in H295R cells. H295R cells were transfected with PELP1 siRNA for 48h. After transfection cells were treated for 24h with IGF-II (100 ng/ml). Western blot analyses of PELP1 and Aromatase were performed on 50 µg of total protein. GAPDH was used as a loading control (A). H295R cells were treated for 6 h with IGF-II (100 ng/ml). Total protein extract were immunoprecipitased with 1 µg of anti-PELP1 antibody. The samples were immunoblotted for SF-1. Protein expression for each sample was normalized to PELP1 content (B). Results are representative of three independent experiments.

14.10 PELP1 knock down reduces cell proliferation in H295R.

In conclusion since PELP1 silencing deprive H295R cells we investigated PELP1 silencing effects on cell proliferation. PELP1 knock down reduced E2 and IGF-II ability to induce cell proliferation (Fig. 14.10).

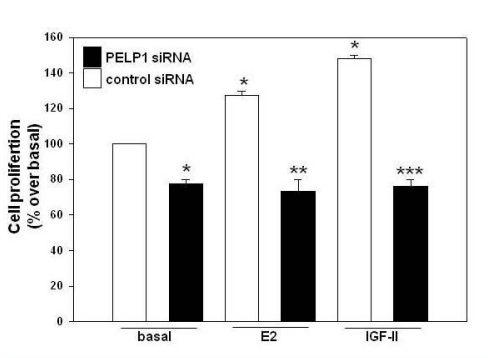


Figure 14.10. PELP1 knock down reduces cell proliferation in H295R. H295R cells were transfected with PELP1 siRNA or a non targeting siRNA (control siRNA). Twenty-four hours after transfection cells were treated for an additional 48 h with IGF-II, E2 or vehicle (basal). (*, P<0.0001 compared with basal; **, P<0.001 compared with E2; ***, P<0.01 compared with IGF-II).

15. Discussion II

The current therapy for adrenocortical carcinoma includes the use of mitotane, a drug with cytotoxic effects controlling steroid secretion, that however shows modest efficacy on metastatic disease (295). In addition, an anti-IGF1R monoclonal antibody has been used in phase-I clinical trial for the treatment of refractory ACC, however no objective responses were seen in the refractory ACC patients (255). The main purpose of this study was to demonstrate the existence of a cross-talk between IGF-II and estrogens and to define the role of PELP1, as a scaffold protein, both in this cross-talk and in controlling cell proliferation, in order to determine if estrogens and/or PELP1 can be targeted to control both E2- and IGF-II-dependent ACC proliferation, giving new indications for ACC therapy.

In the first part of our study we investigated if IGF-II signaling regulates estrogen production in ACC. Our data on human ACC samples demonstrated an increase in the expression of proteins associated with IGF pathways, particularly IGF1R and the phosphorylated form of AKT, but not ERK1/2. A similar expression pattern was found in H295R cells in response to IGF-II treatment. Evaluation of proliferative behavior demonstrated that IGF-II increased cell proliferation, that was blocked by inhibitors for IGF1R, PKC and AKT, but not for ERK1/2, that, do not seem to be involved in IGF-II/IGF1R-dependent adrenal tumor cell proliferation. Moreover, these data indicate H295R cells as a good model for studies investigating the molecular mechanisms involved in ACC proliferation, since they display several features common to the human tumor. In these cells IGF-II increased aromatase expression controlling SF-1 levels and its binding to the PII promoter, the promoter used in H295R cells (191), with a mechanism similar to what we have previously demonstrated in tumor Leydig cells (26). A very recent publication indicated SF-1 staining in 158 of 161 (98%) analyzed ACC, pointing SF-1 as a valuable immunohistochemical marker for this type of tumor (296); our results showing an up-regulation of SF-1 in ACCs are in agreement with this study.

E2 and IGF-I are capable to induce the same genes, including IGF1R, in ER-positive breast cancer cells (297, 298), for this reason the second aim of our study was to evaluate the effects of estrogens on IGF1R expression in ACC. We showed that treatment of H295R

cells with E2 caused an increase in IGF1R mRNA and protein expression. This mechanism was mediated by an increase in CREB phosphorylation and in its binding to IGF1R promoter. This results correlates well with data on ACC, demonstrating increased CREB phosphorylation and IGF1R over-expression, and explain the role played by the increased pCREB levels in ACC. Our data are in agreement with reports indicating the ability of E2 to regulate IGF1R expression in prostate cancer (299). The use of PPT, a specific ERα agonist, confirms the role for this estrogen receptor isoform in mediating estrogen effects on IGF1R. Potentially, E2 controls CREB phosphorylation through a classical mechanism involving protein kinase A (PKA) activation, since we revealed an increase in PKA activity after 1 h stimulation with estradiol (data not shown). E2 was also able to stimulate IGF1R phosphorylation, independently of IGF-II. This was probably due to ERα rapid non-genomic actions (139) directly interacting with the SH2 region of Src, the p85α regulatory subunit of PI3K, Shc and IGF1R (300-302), confirming the ability of E2 to exhibit IGF-II-like activity in ACC. With these preliminary remarks, we aimed to demonstrate the central role played by ERα in the control of IGF-II-dependent ACC cell proliferation. We demonstrated in the examined ACC samples over-expression of cyclin D1, a gene amplified and/or over-expressed in a substantial proportion of different human tumors (303), known to respond to both estrogens and IGFs (289). Particularly, IGF-I is known to stimulate cyclin D1 transcription and enhance cyclin D1 mRNA stability and protein levels (304-306), suggesting a central role for this cell cycle regulator in the control of IGF-dependent tumor proliferation. Herein we demonstrated that ERα, activated by E2, in a ligand dependent, and by IGF-II, in a ligand independent manner, controls cyclin D1 expression. Specifically, IGF-II increased ERα phosphorylation at serine 118 and 167, which results in receptor activation (167, 291, 300). Importantly, as previously shown, we found high ERα levels and demonstrated its phosphorylated status in ACCs. These data highlight the existence of an interaction between IGF-II and ERα in human ACC.

In addition, when ERα was silenced, E2 and IGF-II lost their ability to increase cell proliferation. We also evaluated the ability of an anti IGF1R monoclonal antibody (αIR3), whose specificity is absolute (307), to control E2- and IGF-II-dependent proliferation. αIR3 inhibited basal and IGF-II induced cell proliferation, however little effects were seen on E2-dependent proliferation. Our data indicate that IGF-II dependent proliferative effects in ACC require ERα, highly expressed in ACCs, and possibly explain why IGF1R monoclonal antibodies, recently entered phase-I clinical trials for the therapy of ACC,

failed to give objective responses in refractory ACC patients (255). The existence of a cross-talk between IGF1R and ER signaling pathways was shown *in vitro* in MCF-7 cells where ESR1 silencing resulted in decreased IGF-I-induced G1-S phase progression and decreased expression of CCND1 and CCNE (305). In addition, it was shown *in vivo* in the uterus of ERα knockout mice, that the loss of ERα resulted in IGF-I inability to induce uterine nuclear proliferative responses (308). These studies and our previous data, indicating the ability of antiestrogens to control H295R cell proliferation (190), support the idea of targeting estrogens to interfere with IGF-II signaling in ACC.

In the second phase of the study we focused our attention on Proline-, glutamic acid-, leucine-rich protein-1 (PELP1) (193), that plays important roles in both genomic (195) and nongenomic actions of the ER (196, 197).

PELP1 is localized both in the nuclear and cytoplasmic compartments and has unique functions in both compartments.

Our date indicate that treatment of H295R cells with E2 or IGF-II allowed, only after 10 minutes, the formation of a multiprotein complex formed by PELP1, IGF1R, ERα and Src, probably responsible of PELP1 phosphorylation and activation. In fact PELP1 is shown to be phosphorylated by several kinases including Src (199), PKA (273), HER2 (197), CDKs (309), and its phosphorylation is modulated by estrogen and growth factors (278). Our silencing experiments have shown loss of rapid signals such as MAPK activation. In particular, E2 and IGF-II are able to induce ERK1/2 activation after 10 minutes treatment, and PELP1 silencing blocks E2 and IGF-II induced ERK1/2 activation. This result is in agreement with a previous study showing that PELP1 modulates the interaction of ERs with cSrc, stimulating cSrc enzymatic activity, leading to the activation of the mitogen activated protein kinase (MAPK) pathway (194). These findings implicate that PELP1 acts as a scaffolding protein promoting ER interactions with intracellular kinases and this facilitates activation of ER-nongenomic signaling pathways in adrenocortical carcinoma.

E2 and IGF-II inability to activate MAPK lead to a reduced expression of IGFIR. In fact, it has been shown that estrogens are able to activate IGFIR expression in prostatic cancer cells through ER/ERK/pCREB pathway (288). Moreover, since the enzyme aromatase, responsible of androgen conversion in estrogens, is overexpressed in ACC and estrogens contribute to ACC proliferation activating an autocrine loop (189, 190), and considering

that PELP1 acts as a cofactor able to induce aromatase transcription in breast cancer cells, we also evaluate PELP1 silencing on aromatase expression in H295R cells.

The regulation of aromatase expression is complex and controlled by activation of distinct promoters in a tissue specific manner. Substantial evidence suggests that aromatase promoter II is the main promoter that regulates aromatase expression in H295R cells (191). This promoter contains CRE sites that allow pCREB binding and another site for SF-1 and LRH1 (310). SF-1 and LRH-1 belong to nuclear receptor (NR) family whose transcriptional activity is regulated by coregulator proteins that interact with NRs to modulate NR transactivation functions (311, 312). Our experiments show that PELP1 silencing reduced aromatase expression levels both in basal condition or after stimulation with IGF-II.

This finding correlates with PELP1 and SF-1 ability to immunprecipitate.

A previous study show that PELP1 regulates transcriptional activity of the aromatase gene by recruiting ERRα to a region located between promoter I.3 and II of the *aromatase* gene that controls aromatase expression in breast cancer cells (294). The orphan ERRα is a member of the NR family structurally most related to the canonical ER and has been shown to modulate E2 signaling in some contexts (313). Earlier studies found that ERRα up-regulates aromatase expression via the I.3/II promoters in MCF7 cells (314). This effect was due to PELP1 ability to function as a ERRα cofactor and to enhance aromatase transcription (294).

PELP1 contains several motifs and domains that are commonly present in many transcriptional coactivators, including 10 nuclear receptor (NR)-interacting boxes (LXXLL motifs) (193). Accordingly, PELP1 is shown to interact with and modulate functions of several nuclear receptors and transcriptional activators including AR (205, 315), ERR (294), GR (316, 317), PR (193), RXR (263), FHL2 (205), AP1 (261, 294) and STAT3 (278). Through LXXLL region it could interact also with SF-1 and activate aromatase transcription in ACC. The most relevant result was the effect on cell proliferation. After PELP1 silencing both E2 and IGF-II lost the ability to induce cell proliferation indicating PELP1 as a new target for the control of E2 or IGF-II dependent ACC cell growth..

In conclusion results from this study demonstrate the important role played by ERα in mediating the mitogenic activity of E2 and IGF-II in ACC. Both E2 and IGF-II induce

many comparable responses in H295R cells including activation of IGF1R/AKT signaling and CCND1 expression. Our last set of experiments clearly demonstrates that

PELP1 is the scaffold protein involved in the cross-talk between estrogen and IGF-II pathways;

targeting ERα is effective in controlling E2 and IGF-II dependent cell proliferation, revealing a central role for ERα in the mechanisms controlling ACC cell proliferation;

targeting PEL1 is effective in controlling E2 and IGF-II dependent cell proliferation, since it is involved both in the activation of rapid pathways and in transcriptional regulation mechanisms.

These data support the possibility of using anti-estrogens and/or anti-PELP1 molecules to the purpose of a better controll of adrenocortical carcinoma cell proliferation.

References:

1. Calfee, R., and Fadale, P. (2006) Popular ergogenic drugs and supplements in young athletes. *Pediatrics* **117**, e577-589
2. Evans, N. A. (1997) Gym and tonic: a profile of 100 male steroid users. *British Journal of Sports Medicine* **31**, 54-58
3. Pope, H. G., and Katz, D. L. (1994) Psychiatric and Medical Effects of Anabolic-Androgenic Steroid Use: A Controlled study of 160 athletes. *Arch Gen Psychiatry* **51**, 375-382
4. Wakabayashi, T., Onda, H., Tada, T., Iijima, M., and Itoh, Y. (1984) High incidence of peliosis hepatis in autopsy cases of aplastic anemia with special reference to anabolic steroid therapy. *Acta Pathol Jpn* **34**, 1079-1086
5. Fineschi, V., Baroldi, G., Monciotti, F., Reattelli, L. P., and Turillazzi, E. (2001) Anabolic Steroid Abuse and Cardiac Sudden Death. *Archives of Pathology & Laboratory Medicine* **125**, 253-255
6. Shahidi, N. T. (2001) A review of the chemistry, biological action, and clinical applications of anabolic-androgenic steroids. *Clinical Therapeutics* **23**, 1355-1390
7. Al-Ismail, K., Torreggiani, W., Munk, P., and Nicolaou, S. (2002) Gluteal mass in a bodybuilder: radiological depiction of a complication of anabolic steroid use. *European Radiology* **12**, 1366-1369
8. Dohle, G. R., Smit, M., and Weber, R. F. A. (2003) Androgens and male fertility. *World Journal of Urology* **21**, 341-345
9. Eklöf, A. C., Thurelius, A. M., Garle, M., Rane, A., and Sjöqvist, F. (2003) The anti-doping hot-line, a means to capture the abuse of doping agents in the Swedish society and a new service function in clinical pharmacology. *European Journal of Clinical Pharmacology* **59**, 571-577
10. Socas, L., Zumbado, M., PŠrez-Luzardo, O., Ramos, A., PŠrez, C., Hern…ndez, J. R., and Boada, L. D. (2005) Hepatocellular adenomas associated with anabolic androgenic steroid abuse in bodybuilders: a report of two cases and a review of the literature. *British Journal of Sports Medicine* **39**, e27-e27
11. Bronson, F. H., and Matherne, C. M. (1997) Exposure to anabolic-androgenic steroids shortens life span of male mice. *Medicine & Science in Sports & Exercise* **29**, 615-619
12. Modlinski, R., and Fields, K. B. (2006) The effect of anabolic steroids on the gastrointestinal system, kidneys, and adrenal glands. *Curr Sports Med Rep.* **5**, 104-109
13. Li, J., and Al-Azzawi, F. (2009) Mechanism of androgen receptor action. *Maturitas* **63**, 142-148
14. Singh, R., Artaza, J. N., Taylor, W. E., Gonzalez-Cadavid, N. F., and Bhasin, S. (2003) Androgens Stimulate Myogenic Differentiation and Inhibit Adipogenesis in C3H 10T1/2 Pluripotent Cells through an Androgen Receptor-Mediated Pathway. *Endocrinology* **144**, 5081-5088
15. Bhasin, S., Taylor, W. E., Singh, R., Artaza, J., Sinha-Hikim, I., Jasuja, R., Choi, H., and Gonzalez-Cadavid, N. F. (2003) The mechanisms of androgen

effects on body composition: mesenchymal pluripotent cell as the target of androgen action. *J Gerontol A Biol Sci Med Sci.* **58**, M1103-M1110

16. Singh, R., Artaza, J. N., Taylor, W. E., Braga, M., Yuan, X., Gonzalez-Cadavid, N. F., and Bhasin, S. (2006) Testosterone Inhibits Adipogenic Differentiation in 3T3-L1 Cells: Nuclear Translocation of Androgen Receptor Complex with {beta}-Catenin and T-Cell Factor 4 May Bypass Canonical Wnt Signaling to Down-Regulate Adipogenic Transcription Factors. *Endocrinology* **147**, 141-154
17. Simpson, E. R., Mahendroo, M. S., Means, G. D., Kilgore, M. W., Hinshelwood, M. M., Lorence, S., Amarneh, B., Ito, Y., Fisher, C. R., and Michael, M. D. (1994) Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocrine Reviews* **15**, 342-355
18. Honda, S. i., Harada, N., Ito, S., Takagi, Y., and Maeda, S. (1998) Disruption of Sexual Behavior in Male Aromatase-Deficient Mice Lacking Exons 1 and 2 of thecyp19Gene. *Biochemical and Biophysical Research Communications* **252**, 445-449
19. Robertson, K. M., O'Donnell, L., Simpson, E. R., and Jones, M. E. E. (2002) The Phenotype of the Aromatase Knockout Mouse Reveals Dietary Phytoestrogens Impact Significantly on Testis Function. *Endocrinology* **143**, 2913-2921
20. Faustini-Fustini, M., Rochira, V., and Carani, C. (1999) Oestrogen deficiency in men: where are we today? *European Journal of Endocrinology* **140**, 111-129
21. Fowler, K. A., Gill, K., Kirma, N., Dillehay, D. L., and Tekmal, R. R. (2000) Overexpression of aromatase leads to development of testicular Leydig cell tumors: an in vivo model for hormone-mediated testicular cancer. *American Journal of Pathology* **156**, 347-353
22. Yue, W., Wang, J.-P., Hamilton, C. J., Demers, L. M., and Santen, R. J. (1998) In Situ Aromatization Enhances Breast Tumor Estradiol Levels and Cellular Proliferation. *Cancer Research* **58**, 927-932
23. Tekmal, R. R., Ramachandra, N., Gubba, S., Durgam, V. R., Mantione, J., Toda, K., Shizuta, Y., and Dillehay, D. L. (1996) Overexpression of int-5/aromatase in Mammary Glands of Transgenic Mice Results in the Induction of Hyperplasia and Nuclear Abnormalities. *Cancer Research* **56**, 3180-3185
24. Huseby, R. A. (1976) Estrogen-induced Leydig cell tumor in the mouse model system for the study of carcinogenesis and hormone dependency. *J. Toxicol. Environ. Health.* **1**, 177-192
25. Bosland, M. C. (1996) Hormonal factors in carcinogenesis of the prostate and testis in humans and in animal models. *Prog Clin Biol Res.* **394**, 309-352
26. Sirianni, R., Chimento, A., Malivindi, R., Mazzitelli, I., Ando, S., and Pezzi, V. (2007) Insulin-like growth factor-I, regulating aromatase expression through steroidogenic factor 1, supports estrogen-dependent tumor Leydig cell proliferation. *Cancer Research* **67**, 8368-8377
27. Aquila, S., Sisci, D., Gentile, M., Middea, E., Siciliano, L., and Ando, S. (2002) Human Ejaculated Spermatozoa Contain Active P450 Aromatase. *Journal of Clinical Endocrinology Metabolism* **87**, 3385-3390
28. Aquila, S., Sisci, D., Gentile, M., Carpino, A., Middea, E., Catalano, S., Rago, V., and Ando, S. (2003) Towards a physiological role for cytochrome

P450 aromatase in ejaculated human sperm. *Human Reproduction* **18**, 1650-1659

29. Pezzi, V., Sirianni, R., Chimento, A., Maggiolini, M., Bourguiba, S., Delalande, C., Carreau, S., Ando, S., Simpson, E. R., and Clyne, C. D. (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
30. Sirianni, R., Chimento, A., Ruggiero, C., De Luca, A., Lappano, R., Ando, S., Maggiolini, M., and Pezzi, V. (2008) The Novel Estrogen Receptor, G Protein-Coupled Receptor 30, Mediates the Proliferative Effects Induced by 17{beta}-Estradiol on Mouse Spermatogonial GC-1 Cell Line. *Endocrinology* **149**, 5043-5051
31. Evans, N. A. (1997) Local complications of self administered anabolic steroid injections. *British Journal of Sports Medicine* **31**, 349-350
32. Saez, J. M. (1994) Leydig cells: endocrine, paracrine, and autocrine regulation. *Endocrine Reviews* **15**, 574-626
33. Casella, S. J., Smith, E. P., van Wyk, J. J., Joseph, D. R., Hynes, M. A., Hoyt, E. C., and Lund, P. K. (1987) Isolation of rat testis cDNAs encoding an insulin-like growth factor I precursor. *DNA* **6**, 325-330
34. Liu, J. P., Baker, J., Perkins, A. S., Robertson, E. J., and 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
35. Baker, J., Hardy, M. P., Zhou, J., Bondy, C., Lupu, F., Bellve, A. R., and Efstratiadis, A. (1996) Effects of an Igf1 gene null mutation on mouse reproduction. *Mol Endocrinol.* **10**, 903-918
36. Wang, G. M., O'Shaughnessy, P. J., Chubb, C., Robaire, B., and Hardy, M. P. (2003) Effects of Insulin-Like Growth Factor I on Steroidogenic Enzyme Expression Levels in Mouse Leydig Cells. *Endocrinology* **144**, 5058-5064
37. De Kretser, D. M., and Kerr, J. B. (1994) The cytology of the testis. In *ThePhysiology of Reproduction* Vol. E Knobil & JD Neill. New York: Raven Press pp. 1177-1290
38. Gaytan, F., Bellido, C., Aguilar, E., and 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
39. Gaytan, F., Bellido, C., Morales, C., Reymundo, C., Aguilar, E., and 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
40. Borland, K., Mita, M., Oppenheimer, C. L., Blinderman, L. A., Massague, J., Hall, P. F., and Czech, M. P. (1984) The actions of insulin-like growth factors I and II on cultured Sertoli cells. *Endocrinology* **114**, 240-246
41. Sharpe, R. M. (1994) *Regulation of Spermatogenesis*, ,Raven Press, New York
42. Forest, M. G., De Peretti, E., and Bertrand, J. (1976) Hypothalamic-pituitary-gonadal relationships in man from birth to puberty. . *Clin Endocrinol* **5**, 551-569.

43. Hallmark, N., Walker, M., McKinnell, C., Mahood, I. K., Scott, H., Bayne, R., Coutts, S., Anderson, R. A., Greig, I., Morris, K., and ., e. a. (2007) Effects of monobutyl and di(n-butyl) phthalate in vitro on steroidogenesis and Leydig cell aggregation in fetal testis explants from the rat: comparison with effects in vivo in the fetal rat and neonatal marmoset and in vitro in the human. *Environ Health Perspect* **115**, 390-396
44. Habert, R., Lejeune, H., and Saez, J. M. (2001) Origin, differentiation and regulation of fetal and adult Leydig cells. . *Mol Cell Endocrinol* **179**, 47-74
45. Anderson, R. A., and Sharpe, R. M. (2000) Regulation of inhibin production in the human male and its clinical applications. *Int J Androl* **23**, 136-144
46. Reyes, F. I., Boroditsky, R. S., Winter, J. S., and Faiman, C. (1974) Studies on human sexual development. II. Fetal and maternal serum gonadotropin and sex steroid concentrations. *J Clin Endocrinol Metab* **38**, 612-627.
47. Hadziselimovic, F., Zivkovic, D., Bica, D. T., and Emmons, L. R. (2005) he importance of mini-puberty for fertility in cryptorchidism. *T J Urol* **174**, 1536-1539
48. Forest, M. G., Cathiard, A. M., and Bertrand, J. A. (1973) Evidence of testicular activity in early infancy. *J Clin Endocrinol Metab* **37**, 148-151.
49. Dixson, A. F. (1986) Plasma testosterone concentrations during postnatal development in the male common marmoset. . *Folia Primatol* **47**, 166-170
50. Mann, D. R., and Fraser, H. M. (1996) The neonatal period: a critical interval in male primate development. *J Endocrinol* **149,**, 191-197.
51. Chemes, H. E. (2001) Infancy is not a quiescent period of testicular development. *Int J Androl* **24**, 2-7
52. Kelnar, C. J., McKinnell, C., Walker, M., Morris, K. D., Wallace, W. H., Saunders, P. T., Fraser, H. M., and Sharpe, R. M. (2002) Testicular changes during infantile 'quiescence' in the marmoset and their gonadotrophin dependence: a model for investigating susceptibility of the prepubertal human testis to cancer therapy? *Hum Reprod* **17**, 1367-1378.
53. Plant, T. M. (2006) The male monkey as a model for the study of the neurobiology of puberty onset in man. *Mol Cell Endocrinol* **254-255**, 97-102
54. Dufau, M. L. (1988) Endocrine Regulation and Communicating Functions of the Leydig Cell. *Annual Review of Physiology* **50**, 483-508
55. Cooke, B. A. (1990) Is cyclic AMP an obligatory second messenger for luteinizing hormone? *Molecular and Cellular Endocrinology* **69**, C11-C15
56. Walker, W. H., and Cheng, J. (2005) FSH and testosterone signaling in Sertoli cells. *Reproduction* **130**, 15-28
57. Loosfelt, H., Misrahi, M., Atger, M., Salesse, R., Vu Hai-Luu Thi, M. T., Jolivet, A., Guiochon-Mantel, A., Sar, S., Jallal, B., Garnier, J., and al, e. (1989) Cloning and sequencing of porcine LH-hCG receptor cDNA: variants lacking transmembrane domain. *Science* **245**, 525-528
58. Marshall, G. R., Ramaswamy, S., and Plant, T. M. (2005) Gonadotropin-independent proliferation of the pale type A spermatogonia in the adult rhesus monkey (Macaca mulatta). *Biol Reprod* **73**, 222-229
59. Simorangkir, D. R., Ramaswamy, S., Marshall, G. R., Pohl, C. R., and Plant, T. M. (2009) A selective monotropic elevation of FSH, but not that of LH, amplifies the proliferation and differentiation of spermatogonia in the adult rhesus monkey (Macaca mulatta). *Hum Reprod.*

60. Abel, M. H., Baker, P. J., Charlton, H. M., Monteiro, A., Verhoeven, G., De Gendt, K., Guillou, F., and O'Shaughnessy, P. J. (2008) Spermatogenesis and sertoli cell activity in mice lacking sertoli cell receptors for follicle-stimulating hormone and androgen. *Endocrinology* **149**, 3279-3285
61. Steger, K., Aleithe, I., Behre, H., and Bergmann, M. (1998) The proliferation of spermatogonia in normal and pathological human seminiferous epithelium: an immunohistochemical study using monoclonal antibodies against Ki-67 protein and proliferating cell nuclear antigen. *Mol Hum Reprod* **4**, 227-233.
62. van Alphen, M. M., van de Kant, H. J., and de Rooij, D. G. (1988) Follicle-stimulating hormone stimulates spermatogenesis in the adult monkey. *Endocrinology* **123**, 1449-1455.
63. Singh, J., O'Neill, C., and Handelsman, D. J. (1995) Induction of spermatogenesis by androgens in gonadotropin-deficient (hpg) mice. *Endocrinology* **136**, 5311-5321.
64. Matsumoto, A. M., and Bremner, W. J. (1985) Stimulation of sperm production by human chorionic gonadotropin after prolonged gonadotropin suppression in normal men. *J Androl* **6.**, 137-143
65. Tapanainen, J. S., Aittomaki, K., Min, J., Vaskivuo, T., and Huhtaniemi, I. T. (1997) Men homozygous for an inactivating mutation of the follicle-stimulating hormone (FSH) receptor gene present variable suppression of spermatogenesis and fertility. *Nat Genet* **15**, 205-206
66. Baird, D. T., Galbraith, A., Fraser, I. S., and Newsam, J. E. (1973) The concentration of oestrone and oestradiol-17 in spermatic venous blood in man. *Journal of Endocrinology* **57**, 285-288
67. Freeman, D. A., and Ascoli, M. (1982) Studies on the source of cholesterol used for steroid biosynthesis in cultured Leydig tumor cells. *Journal of Biological Chemistry* **257**, 14231-14238
68. Chung, B. C., Matteson, K. J., Voutilainen, R., Mohandas, T. K., and Miller, W. L. (1986) Human Cholesterol Side-Chain Cleavage Enzyme, P450scc: cDNA Cloning, Assignment of the Gene to Chromosome 15, and Expression in the Placenta. *Proceedings of the National Academy of Sciences* **83**, 8962-8966
69. Pollack, S. E., Furth, E. E., Kallen, C. B., Arakane, F., Kiriakidou, M., Kozarsky, K. F., and Strauss, J. F., III (1997) Localization of the Steroidogenic Acute Regulatory Protein in Human Tissues. *Journal of Clinical Endocrinology Metabolism* **82**, 4243-4251
70. Stocco, D. M., and Clark, B. J. (1996) Regulation of the acute production of steroids in steroidogenic cells. *Endocrine Reviews* **17**, 221-244
71. Clark, B. J., Wells, J., King, S. R., and Stocco, D. M. (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). *Journal of Biological Chemistry* **269**, 28314-28322
72. Lin, D., Sugawara, T., Strauss, J. F., III, Clark, B. J., Stocco, D. M., Saenger, P., Rogol, A., and Miller, W. L. (1995) Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. *Science* **267**, 1828-1831
73. Caron, K. M., Soo, S. C., Wetsel, W. C., Stocco, D. M., Clark, B. J., and Parker, K. L. (1997) Targeted disruption of the mouse gene encoding

steroidogenic acute regulatory protein provides insights into congenital lipoid adrenal hyperplasia. *Proceedings of the National Academy of Sciences* **94**, 11540-11545

74. Weusten, J. J., Smals, A. G., Hofman, J. A., Kloppenborg, P. W., and Benraad, T. J. (1987) Early time sequence in pregnenolone metabolism to testosterone in homogenates of human and rat testis. *Endocrinology* **120**, 1909-1913
75. Matteson, K. J., Picado-Leonard, J., Chung, B. C., Mohandas, T. K., and Miller, W. (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
76. Chung, B. C., Picado-Leonard, J., Haniu, M., Bienkowski, M., Hall, P. F., Shively, J. E., and Miller, W. L. (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. *Proceedings of the National Academy of Sciences* **84**, 407-411
77. Inano, H., Ishii-Ohba, H., Sugimoto, Y., Ohta, Y., Morikawa, T., Yoshida, M., and Tamaoki, B. (1990) Purification and properties of enzymes related to steroid hormone synthesis. *Ann N Y Acad Sci.* **595**, 17-25
78. Luu-The, V., Labrie, C., Simard, J., Lachance, Y., Zhao, H. F., Couet, J., Leblanc, G., and Labrie, F. (1990) Structure of two in tandem human 17 beta-hydroxysteroid dehydrogenase genes. *Molecular Endocrinology* **4**, 268-275
79. Lorence, M. C., Corbin, C. J., Kamimura, N., Mahendroo, M. S., and Mason, J. I. (1990) Structural analysis of the gene encoding human 3 beta-hydroxysteroid dehydrogenase/delta 5----4-isomerase. *Mol Endocrinol.* **4**, 1850-1855
80. Andersson, S., and Russell, D. W. (1990) Structural and Biochemical Properties of Cloned and Expressed Human and Rat Steroid 5 {alpha}-Reductases. *Proceedings of the National Academy of Sciences* **87**, 3640-3644
81. Chen, S. A., Besman, M. J., Sparkes, R. S., Zollman, S., Klisak, I., Mohandas, T., Hall, P. F., and Shively, J. E. (1988) Human aromatase: cDNA cloning, Southern blot analysis, and assignment of the gene to chromosome 15. *DNA.* **7**, 27-38
82. Borer, J. G., Tan, P. E., and Diamond, D. A. (2000) The spectrum of Sertoli cell tumors in children. *Urol Clin North Am.* **27**, 529-541
83. Harms, D., and Kock, L. R. (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
84. Rushton, H. G., and Belman, A. B. (1993) Testis-sparing surgery for benign lesions of the prepubertal testis. *Urol Clin North Am.* **20**, 27-37
85. Kaplan, G. W., Cromie, W. J., Kelalis, P. P., Silber, I., and Tank, E. S., Jr. (1986) Gonadal stromal tumors: a report of the Prepubertal Testicular Tumor Registry. *J Urol.* **136**, 300-302
86. H:, M. (2005) *Testis* Vol. 231-237, Office for National Statistics, London
87. Richiardi, L., Bellocco, R., Adami, H. O., Torrang, A., Barlow, L., Hakulinen, T., Rahu, M., Stengrevics, A., Storm, H., Tretli, S., and al., e. (2004)) Testicular cancer incidence in eight northern European countries: secular and recent trends. *Cancer Epidemiol Biomarkers Prev* **13**, 2157-2166

88. Skakkebaek, N. E. (1972) Abnormal morphology of germ cells in two infertile men. *Acta Pathol Microbiol Scand A* **80**, 374-378
89. Rajpert-De Meyts, E. (2006) Developmental model for the pathogenesis of testicular carcinoma in situ: genetic and environmental aspects. . *Hum Reprod Update* **12**, 303-323
90. Holm, M., Rajpert-De Meyts, E., Andersson, A. M., and Skakkebaek, N. E. (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
91. Shenker, A., Laue, L., Kosugi, S., Merendino, J. J., Minegishi, T., and Cutler, G. B. (1993) A constitutively activating mutation of the luteinizing hormone receptor in familial male precocious puberty. *Nature* **365**, 652-654
92. Liu, G., Duranteau, L., Carel, J. C., Monroe, J., Doyle, D. A., and Shenker, A. (1999) Leydig-Cell Tumors Caused by an Activating Mutation of the Gene Encoding the Luteinizing Hormone Receptor. *The New England Journal of Medicine* **341**, 1731-1736
93. Iiri, T., Herzmark, P., Nakamoto, J. M., Van Dop, C., and Bourne, H. R. (1994) Rapid GDP release from Gs[alpha] in patients with gain and loss of endocrine function. *Nature* **371**, 164-168
94. Villares Fragoso, M. C., Latronico, A. C., Carvalho, F. M., Zerbini, M. C., Marcondes, J. A. M., Araujo, L. M. B., Lando, V. S., Frazzatto, E. T., Mendonca, B. B., and Villares, S. M. (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
95. Themmen, A. P., and Brunner, H. G. (1996) Luteinizing hormone receptor mutations and sex differentiation. *Eur J Endocrinol.* **134**, 533-540
96. Dilworth, J. P., Farrow, G. M., and Oesterling, J. E. (1991) Non-germ cell tumors of testis. *Urology* **37**, 399-417
97. Rich, M. A., and Keating, M. A. (2000) Leydig cell tumors and tumors associated with congenital adrenal hyperplasia. *Urol Clin North Am.* **27**, 519-528
98. Kim, I., Young, R. H., and Scully, R. E. (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
99. Bertram, K. A., Bratloff, B., Hodges, G. F., and Davidson, H. (1991) Treatment of malignant Leydig cell tumor. *Cancer* **68**, 2324-2329
100. Cheville, J. C., Sebo, T. J., Lager, D. J., Bostwick, D. G., and Farrow, G. M. (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
101. Iwata, H., Hirouchi, Y., and Koike, Y. (1991) Historical control data of non-neoplastic and neoplastic lesions in F-344/DuCrj. *J Appl Toxicol* **4**, 24
102. Boorman, G., Eustis, S., Montgomery, C., Elwell, M., and Mackenzie, W. (1990) Pathology of the Fisher rat. *Academic Press, San Diego*, 405-418
103. Jones, T. C., Mohr, U., and Hunt, R. D. (1987) Monographs on phatology of laboratory animals Genital System. *Spring -Verlag, Berlin*, 184-192

104. Carpino, A., Rago, V., Pezzi, V., Carani, C., and Ando, S. (2007) Detection of aromatase and estrogen receptors (ER{alpha}, ER{beta}1, ER{beta}2) in human Leydig cell tumor. *European Journal of Endocrinology* **157**, 239-244
105. Düe, W., Dieckmann, K., Loy, V., and 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
106. Turner, K. J., Macpherson, S., Millar, M. R., McNeilly, A. S., Williams, K., Cranfield, M., Groome, N. P., Sharpe, R. M., Fraser, H. M., and Saunders, P. T. (2002) Development and validation of a new monoclonal antibody to mammalian aromatase. *Journal of Endocrinology* **172**, 21-30
107. Ho, G. T., Gardner, H., Mostofi, K., DeWolf, W. C., Loughlin, K. R., and Morgentaler, A. (1994) The effect of testicular nongerm cell tumors on local spermatogenesis. *Fertil Steril.* **62**, 162-166
108. Saunders, P. T. K., Sharpe, R. M., Williams, K., Macpherson, S., Urquart, H., Irvine, D. S., and Millar, M. R. (2001) Differential expression of oestrogen receptor {{alpha}} and {beta} proteins in the testes and male reproductive system of human and non-human primates. *Molecular Human Reproduction* **7**, 227-236
109. Saunders, P. T. K., Millar, M. R., Macpherson, S., Irvine, D. S., Groome, N. P., Evans, L. R., Sharpe, R. M., and Scobie, G. A. (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
110. Akingbemi, B. (2005) Estrogen regulation of testicular function. *Reproductive Biology and Endocrinology* **3**, 51-63
111. Carreau, S., Silandre, D., Bourguiba, S., Hamden, K., Said, L., Lambard, S., Galeraud-Denis, I., and Delalande, C. (2007) Estrogens and male reproduction: a new concept. *Braz J Med Biol Res.* **40**, 761-768
112. Sebastian, S., and Bulun, S. E. (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
113. Simpson, E. R., Clyne, C., Rubin, G., Boon, W. C., Robertson, K., Britt, K., Speed, C., and Jones, M. (2002) Aromatase: a brief overview. *Annu Rev Physiol.* **64**, 93-127
114. Graddy, L. G., Kowalski, A. A., Simmen, F. A., Davis, S. L. F., Baumgartner, W. W., and Simmen, R. C. M. (2000) Multiple isoforms of porcine aromatase are encoded by three distinct genes. *The Journal of Steroid Biochemistry and Molecular Biology* **73**, 49-57
115. Kishida, M., and Callard, G. V. (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
116. Conley, A., and Hinshelwood, M. (2001) Mammalian aromatases. *Reproduction* **121**, 685-695
117. Means, G. D., Kilgore, M. W., Mahendroo, M. S., Mendelson, C. R., and Simpson, E. R. (1991) Tissue-specific promoters regulate aromatase

cytochrome P450 gene expression in human ovary and fetal tissues. *Mol Endocrinol.* **5**, 2005-2013

118. Birk, O. S., Casiano, D. E., Wassif, C. A., Cogliati, T., Zhao, L., Zhao, Y., Grinberg, A., Huang, S., Kreidberg, J. A., Parker, K. L., Porter, F. D., and Westphal, H. (2000) The LIM homeobox gene Lhx9 is essential for mouse gonad formation. *Nature* **403**, 909-913
119. Hickey, G. J., Krasnow, J. S., Beattie, W. G., and Richards, J. S. (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. *Molecular Endocrinology* **4**, 3-12
120. Young, M., and McPhaul, M. J. (1998) A Steroidogenic Factor-1-binding site and cyclic adenosine 3',5'-monophosphate response element-lke elements are required for the activity of the rat aromatase promoter in rat Leydig tumor cell lines. *Endocrinology* **139**, 5082-5093
121. Wang, Z. J., Jeffs, B., Ito, M., Achermann, J. C., Yu, R. N., Hales, D. B., and Jameson, J. L. (2001) Aromatase (Cyp19) expression is up-regulated by targeted disruption of Dax1. *Proceedings of the National Academy of Sciences* **98**, 7988-7993
122. Silandre, D., Delalande, C., Durand, P., and Carreau, S. (2007) Three promoters PII, PI.f, and PI.tr direct the expression of aromatase (cyp19) gene in male rat germ cells. *Journal of Molecular Endocrinology* **39**, 169-181
123. Teixeira, C. V., Silandre, D., de Souza Santos, A. M., Delalande, C., Sampaio, F. J., Carreau, S., and da Fonte Ramos, C. (2007) Effects of maternal undernutrition during lactation on aromatase, estrogen, and androgen receptors expression in rat testis at weaning. *Journal of Endocrinology* **192**, 301-311
124. Hamden, K., Silandre, D., Delalande, C., El Feki, A., and Carreau, S. (2008) Age-related decrease in aromatase and estrogen receptor (ER[alpha] and ER[beta]) expression in rat testes: protective effect of low caloric diets. *Asian J Androl* **10**, 177-187
125. Haus-Seuffert, P., and Meisterernst, M. (2000) Mechanisms of transcriptional activation of cAMP-responsive element-binding protein CREB. *Molecular and Cellular Biochemistry* **212**, 5-9
126. PFAHL, M. (1993) Nuclear Receptor/AP-1 Interaction. *Endocrine Reviews* **14**, 651-658
127. Luo, X., Ikeda, Y., and Parker, K. L. (1994) A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. *Cell* **77**, 481-490
128. Gruber, C. J., Tschugguel, W., Schneeberger, C., and Huber, J. C. (2002) Production and Actions of Estrogens. *The New England Journal of Medicine* **346**, 340-352
129. Kraus, W. L., McInerney, E. M., and Katzenellenbogen, B. S. (1995) Ligand-Dependent, Transcriptionally Productive Association of the Amino- and Carboxyl-Terminal Regions of a Steroid Hormone Nuclear Receptor. *Proceedings of the National Academy of Sciences* **92**, 12314-12318
130. McInerney, E. M., Tsai, M. J., O'Malley, B. W., and Katzenellenbogen, B. S. (1996) Analysis of estrogen receptor transcriptional enhancement by a

nuclear hormone receptor coactivator. *Proceedings of the National Academy of Sciences* **93**, 10069-10073
131. Giguere, V. (1999) Orphan Nuclear Receptors: From Gene to Function. *Endocrine Reviews* **20**, 689-725
132. Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., Enmark, E., Pettersson, K., Warner, M., and Gustafsson, J. A. (2001) Mechanisms of Estrogen Action. *Physiological Reviews* **81**, 1535-1565
133. Chang, W. Y., and Prins, G. S. (1999) Estrogen receptor-beta: implications for the prostate gland. *Prostate.* **40**, 115-124
134. Kuiper, G. G. J. M., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., and Gustafsson, J. A. (1997) Comparison of the Ligand Binding Specificity and Transcript Tissue Distribution of Estrogen Receptors {alpha} and {beta}. *Endocrinology* **138**, 863-870
135. Kuiper, G. G. J. M., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van der Saag, P. T., van der Burg, B., and Gustafsson, J. A. (1998) Interaction of Estrogenic Chemicals and Phytoestrogens with Estrogen Receptor {beta}. *Endocrinology* **139**, 4252-4263
136. Hall, J. M., and McDonnell, D. P. (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
137. Hewitt, S. C., and Korach, K. S. (2002) Estrogen receptors: structure, mechanisms and function. *Rev Endocr Metab Disord.* **3**, 193-200
138. Loomis, A. K., and 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. *Biology of Reproduction* **62**, 995-1004
139. Levin, E. R. (2005) Integration of the Extranuclear and Nuclear Actions of Estrogen. *Molecular Endocrinology* **19**, 1951-1959
140. Levin, E. R. (2009) Plasma membrane estrogen receptors. *Trends in Endocrinology & Metabolism* **20**, 477-482
141. Levin, E. R. (2008) Rapid signaling by steroid receptors. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **295**, R1425-R1430
142. Bjornstrom, L., and Sjoberg, M. (2005) Mechanisms of Estrogen Receptor Signaling: Convergence of Genomic and Nongenomic Actions on Target Genes. *Molecular Endocrinology* **19**, 833-842
143. Hewitt, S. C., Deroo, B. J., and Korach, K. S. (2005) Signal transduction. A new mediator for an old hormone? *Science* **307**, 1572-1573
144. Song, R. X. D., Zhang, Z., and Santen, R. J. (2005) Estrogen rapid action via protein complex formation involving ERalpha and Src. *Trends in Endocrinology and Metabolism* **16**, 347-353
145. Song, R. X. D., and Santen, R. J. (2006) Membrane Initiated Estrogen Signaling in Breast Cancer. *Biology of Reproduction* **75**, 9-16
146. Manavathi, B., and Kumar, R. (2006) Steering estrogen signals from the plasma membrane to the nucleus: two sides of the coin. *J Cell Physiol* **207**, 594-604

147. Harrington, W. R., Kim, S. H., Funk, C. C., Madak-Erdogan, Z., Schiff, R., Katzenellenbogen, J. A., and Katzenellenbogen, B. S. (2006) Estrogen Dendrimer Conjugates that Preferentially Activate Extranuclear, Nongenomic Versus Genomic Pathways of Estrogen Action. *Molecular Endocrinology* **20**, 491-502
148. Pedram, A., Razandi, M., and Levin, E. R. (2006) Nature of Functional Estrogen Receptors at the Plasma Membrane. *Molecular Endocrinology* **20**, 1996-2009
149. Filardo, E. J., Quinn, J. A., Frackelton, A. R., Jr., and Bland, K. I. (2002) Estrogen action via the G Protein-Coupled Receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the Epidermal Growth Factor Receptor-to-MAPK signaling axis. *Molecular Endocrinology* **16**, 70-84
150. Maggiolini, M., Vivacqua, A., Fasanella, G., Recchia, A. G., Sisci, D., Pezzi, V., Montanaro, D., Musti, A. M., Picard, D., and Ando, S. (2004) The G Protein-coupled Receptor GPR30 Mediates c-fos Up-regulation by 17beta-Estradiol and Phytoestrogens in Breast Cancer Cells. *Journal of Biological Chemistry* **279**, 27008-27016
151. Revankar, C. M., Cimino, D. F., Sklar, L. A., Arterburn, J. B., and Prossnitz, E. R. (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* **307**, 1625-1630
152. Prossnitz, E. R., and Maggiolini, M. (2009) Mechanisms of estrogen signaling and gene expression via GPR30. *Molecular and Cellular Endocrinology* **308**, 32-38
153. Sakamoto, H., Matsuda, K. i., Hosokawa, K., Nishi, M., Morris, J. F., Prossnitz, E. R., and Kawata, M. (2007) Expression of GPR30, a G protein-coupled membrane estrogen receptor, in oxytocin neurons of the rat paraventricular and supraoptic nuclei. *Endocrinology* **148**, 5842-5850
154. Chagin, A. S., and Savendahl, L. (2007) GPR30 estrogen receptor expression in the growth plate declines as puberty progresses. *Journal of Clinical Endocrinology Metabolism* **92**, 4873-4877
155. Wang, C., Prossnitz, E. R., and Roy, S. K. (2007) Expression of G Protein-Coupled Receptor 30 in the hamster ovary: differential regulation by gonadotropins and steroid hormones. *Endocrinology* **148**, 4853-4864
156. Brailoiu, E., Dun, S. L., Brailoiu, G. C., Mizuo, K., Sklar, L. A., Oprea, T. I., Prossnitz, E. R., and Dun, N. J. (2007) Distribution and characterization of estrogen receptor G protein-coupled receptor 30 in the rat central nervous system. *Journal of Endocrinology* **193**, 311-321
157. Funakoshi, T., Yanai, A., Shinoda, K., Kawano, M. M., and Mizukami, Y. (2006) G protein-coupled receptor 30 is an estrogen receptor in the plasma membrane. *Biochemical and Biophysical Research Communications* **346**, 904-910
158. Filardo, E. J., Quinn, J. A., Bland, K. I., and Frackelton, A. R., Jr. (2000) Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the Epidermal Growth Factor Receptor through release of HB-EGF. *Molecular Endocrinology* **14**, 1649-1660

159. Isensee, J., Meoli, L., Zazzu, V., Nabzdyk, C., Witt, H., Soewarto, D., Effertz, K., Fuchs, H., Gailus-Durner, V., Busch, D., Adler, T., de Angelis, M. H., Irgang, M., Otto, C., and Noppinger, P. R. (2009) Expression Pattern of G Protein-Coupled Receptor 30 in LacZ Reporter Mice. *Endocrinology* **150**, 1722-1730
160. Chimento, A., Sirianni, R., Delalande, C., Silandre, D., Bois, C., And•, S., Maggiolini, M., Carreau, S., and Pezzi, V. (2010) 17β-estradiol activates rapid signaling pathways involved in rat pachytene spermatocytes apoptosis through GPR30 and ERα. *Mol Cell Endocrinol.* **320**, 136-144
161. Chimento, A., Sirianni, R., Zolea, F., Bois, C., Delalande, C., And•, S., Maggiolini, M., Aquila, S., Carreau, S., and Pezzi, V. (2011) Gper and ESRs are expressed in rat round spermatids and mediate oestrogen-dependent rapid pathways modulating expression of cyclin B1 and Bax. *International Journal of Andrology* **In press**
162. Lucas, T. s. F. G., Royer, C., Siu, E. R., Lazari, M. F., and Porto, C. S. (2010) Expression and Signaling of G Protein-Coupled Estrogen Receptor 1 (GPER) in Rat Sertoli Cells. *Biology of Reproduction* **83**, 307-317
163. O'Donnell, L., Robertson, K. M., Jones, M. E., and Simpson, E. R. (2001) Estrogen and spermatogenesis. *Endocrine Reviews* **22**, 289-318
164. Lephart, E. D., and Simpson, E. R. (1991) Assay of aromatase activity. *Methods Enzymol.* **206**, 477-483
165. Bradford, M. M. (1976) A rapid and sensitive method for thequantitation of microgram quantities of protein utilizing theprinciple of protein-dye binding. *Analytical Biochemistry* **72**, 248-254
166. Sylvester, P. W. (2011) Optimization of the Tetrazolium Dye (MTT) Colorimetric Assay for Cellular Growth and Viability. In *Drug Design and Discovery* (Satyanarayanajois, S. D. D., ed) Vol. 716 pp. 157-168, Humana Press
167. Bunone, G., Briand, P. A., Miksicek, R. J., and Picard, D. (1996) Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO J.* **15**, 2174-2183
168. Tora, L., Mullick, A., Metger, D., Ponglikitmongkol, M., Park, I., and Chambon, P. (1989) The cloned human oestrogen receptor contains a mutation which alters its hormone binding properties. *EMBO J* **8**, 1981-1986
169. Foradori, C. D., Weiser, M. J., and Handa, R. J. (2008) Non-genomic actions of androgens. *Frontiers in Neuroendocrinology* **29**, 169-181
170. Maggiolini, M., DonzŠ, O., Jeannin, E., And•, S., and Picard, D. (1999) Adrenal Androgens Stimulate the Proliferation of Breast Cancer Cells as Direct Activators of Estrogen Receptor à. *Cancer Research* **59**, 4864-4869
171. Carreau, S., Delalande, C., Silandre, D., Bourguiba, S., and Lambard, S. (2006) Aromatase and estrogen receptors in male reproduction. *Molecular and Cellular Endocrinology* **246**, 65-68
172. Bhasin, S., Woodhouse, L., Casaburi, R., Singh, A. B., Bhasin, D., Berman, N., Chen, X., Yarasheski, K. E., Magliano, L., Dzekov, C., Dzekov, J., Bross, R., Phillips, J., Sinha-Hikim, I., Shen, R., and Storer, T. W. (2001) Testosterone dose-response relationships in healthy young men. *AJP - Endocrinology and Metabolism* **281**, E1172-E1181

173. Bhasin, S., Woodhouse, L., Casaburi, R., Singh, A. B., Mac, R. P., Lee, M., Yarasheski, K. E., Sinha-Hikim, I., Dzekov, C., Dzekov, J., Magliano, L., and Storer, T. W. (2005) Older Men Are as Responsive as Young Men to the Anabolic Effects of Graded Doses of Testosterone on the Skeletal Muscle. *Journal of Clinical Endocrinology Metabolism* **90**, 678-688
174. Rochefort, H., and Garcia, M. (1983) The estrogenic and antiestrogenic activities of androgens in female target tissues. *Pharmacology & Therapeutics* **23**, 193-216
175. Le, H., Arnold, J. T., McFann, K. K., and Blackman, M. R. (2006) DHT and testosterone, but not DHEA or E2, differentially modulate IGF-I, IGFBP-2, and IGFBP-3 in human prostatic stromal cells. *American Journal of Physiology - Endocrinology And Metabolism* **290**, E952-E960
176. Larsson, O., Girnita, A., and Girnita, L. (2005) Role of insulin-like growth factor 1 receptor signalling in cancer. *Br J Cancer* **92**, 2097-2101
177. Prall, O. W. J., Sarcevic, B., Musgrove, E. A., Watts, C. K. W., and Sutherland, R. L. (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. *Journal of Biological Chemistry* **272**, 10882-10894
178. Ma, Q. L., Yang, T. L., Yin, J. Y., Peng, Z. Y., Yu, M., Liu, Z. Q., and Chen, F. P. (2009) Role of insulin-like growth factor-1 (IGF-1) in regulating cell cycle progression. *Biochemical and Biophysical Research Communications* **389**, 150-155
179. Grumbach, M. M., Biller, B. M. K., Braunstein, G. D., Campbell, K. K., Carney, J. A., Godley, P. A., Harris, E. L., Lee, J. K. T., Oertel, Y. C., Posner, M. C., Schlechte, J. A., and Wieand, H. S. (2003) Management of the Clinically Inapparent Adrenal Mass ("Incidentaloma"). *Annals of Internal Medicine* **138**, 424-429
180. Libe, R., Fratticci, A., and Bertherat, J. (2007) Adrenocortical cancer: pathophysiology and clinical management. *Endocrine-Related Cancer* **14**, 13-28
181. Schteingart, D. E. (2001) Current Perspective in the Diagnosis and Treatment of Adrenocortical Carcinoma. *Reviews in Endocrine & Metabolic Disorders* **2**, 323-333
182. Soon, P. S. H., Libe, R., Benn, D. E., Gill, A., Shaw, J., Sywak, M. S., Groussin, L., Bertagna, X., Gicquel, C., Bertherat, J., McDonald, K. L., Sidhu, S. B., and Robinson, B. G. (2008) Loss of Heterozygosity of 17p13, With Possible Involvement of ACADVL and ALOX15B, in the Pathogenesis of Adrenocortical Tumors. *Annals of Surgery* **247**
183. Giordano, T. J., Kuick, R., Else, T., Gauger, P. G., Vinco, M., Bauersfeld, J., Sanders, D., Thomas, D. G., Doherty, G., and Hammer, G. (2009) Molecular Classification and Prognostication of Adrenocortical Tumors by Transcriptome Profiling. *Clinical Cancer Research* **15**, 668-676
184. Bertherat, J., and Bertagna, X. (2009) Pathogenesis of adrenocortical cancer. *Best Practice & Research Clinical Endocrinology & Metabolism* **23**, 261-271
185. Logie, A., Boulle, N., Gaston, V., Perin, L., Boudou, P., Le Bouc, Y., and Gicquel, C. (1999) Autocrine role of IGF-II in proliferation of human

adrenocortical carcinoma NCI H295R cell line. *Journal of Molecular Endocrinology* **23**, 23-32
186. Fottner, C., Hoeflich, A., Wolf, E., and Weber, M. M. (2004) Role of the Insulin-like Growth Factor System in Adrenocortical Growth Control and Carcinogenesis. *Horm Metab Res* **36**, 397-405
187. Barzon, L., Sonino, N., Fallo, F., Palu, G., and Boscaro, M. (2003) Prevalence and natural history of adrenal incidentalomas. *European Journal of Endocrinology* **149**, 273-285
188. Hsing, A. W., Nam, J. M., Co Chien, H. T., McLaughlin, J. K., and Fraumeni, J. F., Jr. (1996) Risk factors for adrenal cancer: an exploratory study. *Int J Cancer* **65**, 432-436
189. Barzon, L., Masi, G., Pacenti, M., Trevisan, M., Fallo, F., Remo, A., Martignoni, G., Montanaro, D., Pezzi, V., and Pal—, G. (2008) Expression of aromatase and estrogen receptors in human adrenocortical tumors. *Virchows Archiv* **452**, 181-191
190. Montanaro, D., Maggiolini, M., Recchia, A. G., Sirianni, R., Aquila, S., Barzon, L., Fallo, F., Ando, S., and Pezzi, V. (2005) Antiestrogens upregulate estrogen receptor {beta} expression and inhibit adrenocortical H295R cell proliferation. *Journal of Molecular Endocrinology* **35**, 245-256
191. Nicol, M. R., Papacleovoulou, G., Evans, D. B., Penning, T. M., Strachan, M. W. J., Advani, A., Johnson, S. J., Quinton, R., and Mason, J. I. (2009) Estrogen biosynthesis in human H295 adrenocortical carcinoma cells. *Molecular and Cellular Endocrinology* **300**, 115-120
192. McKenna, N. J., Lanz, R. B., and O'Malley, B. W. (1999) Nuclear Receptor Coregulators: Cellular and Molecular Biology. *Endocrine Reviews* **20**, 321-344
193. Vadlamudi, R. K., Wang, R. A., Mazumdar, A., Kim, Y. s., Shin, J., Sahin, A., and Kumar, R. (2001) Molecular Cloning and Characterization of PELP1, a Novel Human Coregulator of Estrogen Receptor +Ý. *Journal of Biological Chemistry* **276**, 38272-38279
194. Cheskis, B. J., Greger, J., Cooch, N., McNally, C., McLarney, S., Lam, H. S., Rutledge, S., Mekonnen, B., Hauze, D., Nagpal, S., and Freedman, L. P. (2008) MNAR plays an important role in ERa activation of Src/MAPK and PI3K/Akt signaling pathways. *Steroids* **73**, 901-905
195. Nair, S. S., Mishra, S. K., Yang, Z., Balasenthil, S., Kumar, R., and Vadlamudi, R. K. (2004) Potential Role of a Novel Transcriptional Coactivator PELP1 in Histone H1 Displacement in Cancer Cells. *Cancer Research* **64**, 6416-6423
196. Barletta, F., Wong, C. W., McNally, C., Komm, B. S., Katzenellenbogen, B., and Cheskis, B. J. (2004) Characterization of the Interactions of Estrogen Receptor and MNAR in the Activation of cSrc. *Molecular Endocrinology* **18**, 1096-1108
197. Vadlamudi, R. K., Manavathi, B., Balasenthil, S., Nair, S. S., Yang, Z., Sahin, A. A., and Kumar, R. (2005) Functional Implications of Altered Subcellular Localization of PELP1 in Breast Cancer Cells. *Cancer Research* **65**, 7724-7732
198. Vadlamudi, R. K., Balasenthil, S., Broaddus, R. R., Gustafsson, J. A., and Kumar, R. (2004) Deregulation of Estrogen Receptor Coactivator Proline-,

Glutamic Acid-, and Leucine-Rich Protein-1/Modulator of Nongenomic Activity of Estrogen Receptor in Human Endometrial Tumors. *Journal of Clinical Endocrinology Metabolism* **89**, 6130-6138

199. Greger, J. G., Fursov, N., Cooch, N., McLarney, S., Freedman, L. P., Edwards, D. P., and Cheskis, B. J. (2007) Phosphorylation of MNAR Promotes Estrogen Activation of Phosphatidylinositol 3-Kinase. *Molecular and Cellular Biology* **27**, 1904-1913
200. Balasenthil, S., and Vadlamudi, R. K. (2003) Functional Interactions between the Estrogen Receptor Coactivator PELP1/MNAR and Retinoblastoma Protein. *Journal of Biological Chemistry* **278**, 22119-22127
201. Habashy, H., Powe, D., Rakha, E., Ball, G., Macmillan, R., Green, A., and Ellis, I. (2010) The prognostic significance of PELP1 expression in invasive breast cancer with emphasis on the ER-positive luminal-like subtype. *Breast Cancer Research and Treatment* 3-603
202. Kumar, R., Zhang, H., Holm, C., Vadlamudi, R. K., Landberg, G., and Rayala, S. K. (2009) Extranuclear Coactivator Signaling Confers Insensitivity to Tamoxifen. *Clinical Cancer Research* **15**, 4123-4130
203. Rajhans, R., Nair, S., Holden, A. H., Kumar, R., Tekmal, R. R., and Vadlamudi, R. K. (2007) Oncogenic Potential of the Nuclear Receptor Coregulator Proline-, Glutamic Acid-, Leucine-Rich Protein 1/Modulator of the Nongenomic Actions of the Estrogen Receptor. *Cancer Research* **67**, 5505-5512
204. Dimple, C., Nair, S. S., Rajhans, R., Pitcheswara, P. R., Liu, J., Balasenthil, S., Le, X. F., Burow, M. E., Auersperg, N., Tekmal, R. R., Broaddus, R. R., and Vadlamudi, R. K. (2008) Role of PELP1/MNAR Signaling in Ovarian Tumorigenesis. *Cancer Research* **68**, 4902-4909
205. Nair, S. S., Guo, Z., Mueller, J. M., Koochekpour, S., Qiu, Y., Tekmal, R. R., Schule, R., Kung, H. J., Kumar, R., and Vadlamudi, R. K. (2007) Proline-, Glutamic Acid-, and Leucine-Rich Protein-1/Modulator of Nongenomic Activity of Estrogen Receptor Enhances Androgen Receptor Functions through LIM-Only Coactivator, Four-and-a-Half LIM-Only Protein 2. *Molecular Endocrinology* **21**, 613-624
206. Migliaccio, A., Di Domenico, M., Castoria, G., Nanayakkara, M., Lombardi, M., de Falco, A., Bilancio, A., Varricchio, L., Ciociola, A., and Auricchio, F. (2005) Steroid receptor regulation of epidermal growth factor signaling through Src in breast and prostate cancer cells: steroid antagonist action. *Cancer Research* **65**, 10585-10593
207. Manavathi, B., Nair, S. S., Wang, R. A., Kumar, R., and Vadlamudi, R. K. (2005) Proline-, Glutamic Acid-, and Leucine-Rich Protein-1 Is Essential in Growth Factor Regulation of Signal Transducers and Activators of Transcription 3 Activation. *Cancer Research* **65**, 5571-5577
208. PM, S. (2008) *Williams textbook of endocrinology. 11th ed. ,* Saunders Elservier, Philadelphia
209. Netter, F. H. (1965) *Endocrine system and selected metabolic diseases, in Ciba collection of medical illustration*
210. Mesiano, S., and Jaffe, R. B. (1997) Developmental and Functional Biology of the Primate Fetal Adrenal Cortex. *Endocrine Reviews* **18**, 378-403
211. Goglia, G. *Embriologia Umana II edizione*

212. Miller WL, A. J., Fluck CE (2008) *Pediatric endocrinology. 3rd ed*, Saunders Elservier, Philadelphia
213. Shima, S., Mitsunaga, M., and Nakao, T. (1972) Effect of ACTH on cholesterol dynamics in rat adrenal tissue. *Endocrinology* **90**, 808-814
214. Miller, W. (2009) Androgen synthesis in adrenarche. *Reviews in Endocrine & Metabolic Disorders* **10**, 3-17
215. Beins, D. M., Vining, R., and Balasubramaniam, S. (1982) Regulation of neutral cholesterol esterase and acyl-CoA : cholesterol acyltransferase in the rat adrenal gland. *Biochem J* **202**, 631-637
216. Miller, W. L. (1988) Molecular biology of steroid hormone synthesis. *Endocrine Reviews* **9**, 295-318
217. Bose, H. S., Whittal, R. M., Baldwin, M. A., and Miller, W. L. (1999) The active form of the steroidogenic acute regulatory protein, StAR, appears to be a molten globule. *Proceedings of the National Academy of Sciences* **96**, 7250-7255
218. Morohashi, K., Sogawa, K., Omura, T., and Fujii-Kuriyama, Y. (1987) Gene structure of human cytochrome P-450(SCC), cholesterol desmolase. *J Biochem* **101**, 879-887
219. Lambeth, J. D., and Pember, S. O. (1983) Cytochrome P-450scc-adrenodoxin complex. Reduction properties of the substrate-associated cytochrome and relation of the reduction states of heme and iron-sulfur centers to association of the proteins. *Journal of Biological Chemistry* **258**, 5596-5602
220. Kimura, T., and Suzuki, K. (1967) Components of the Electron Transport System in Adrenal Steroid Hydroxylase. Isolation and properties of non-heme iron protein (adrenodoxin). *Journal of Biological Chemistry* **242**, 485-491
221. Kawainoto, T., Mitsuuchi, Y., Ohnishi, T., Ichikawa, Y., Yokoyama, Y., Sumimoto, H., Toda, K., Miyahara, K., Kuribayashi, I., Nakao, K., and et al. (1990) Cloning and expression of a cDNA for human cytochrome P-450aldo as related to primary aldosteronism. *Biochem Biophys Res Commun* **173**, 309-316
222. Malee, M. P., and Mellon, S. H. (1991) Zone-Specific Regulation of Two Messenger RNAs for P450c11 in the Adrenals of Pregnant and Nonpregnant Rats. *Proceedings of the National Academy of Sciences* **88**, 4731-4735
223. Berube, D., Luu The, V., Lachance, Y., Gagne, R., and Labrie, F. (1989) Assignment of the human 3 beta-hydroxysteroid dehydrogenase gene (HSDB3) to the p13 band of chromosome 1. *Cytogenet Cell Genet* **52**, 199-200
224. Allolio, B., and Fassnacht, M. (2006) Adrenocortical Carcinoma: Clinical Update. *Journal of Clinical Endocrinology Metabolism* **91**, 2027-2037
225. Mundschenk, J., and Lehnert, H. (1998) Malignant pheochromocytoma. *Exp Clin Endocrinol Diabetes* **106**, 373,376
226. Hutter, A. M., and Kayhoe, D. E. (1966) Adrenal cortical carcinoma: Results of treatment with o,p'DDD in 138 patients. *The American journal of medicine* **41**, 581-592
227. Kopf, D., Goretzki, P. E., and Lehnert, H. (2001) Clinical management of malignant adrenal tumors. *Journal of Cancer Research and Clinical Oncology* **127**, 143-155

228. Wooten, M. D., and King, D. K. (1993) Adrenal cortical carcinoma. Epidemiology and treatment with mitotane and a review of the literature. *Cancer* **72**, 3145-3155
229. Harrison, L. E., Gaudin, P. B., and Brennan, M. F. (1999) Pathologic Features of Prognostic Significance for Adrenocortical Carcinoma After Curative Resection. *Arch Surg* **134**, 181-185
230. Ilvesmaki, V., Kahri, A. I., Miettinen, P. J., and Voutilainen, R. (1993) Insulin-like growth factors (IGFs) and their receptors in adrenal tumors: high IGF-II expression in functional adrenocortical carcinomas. *Journal of Clinical Endocrinology Metabolism* **77**, 852-858
231. Sasano H, S. T., Shizawa S, Kato K, Nagura H (194) Transforming growth factor alpha, epidermal growth factor, and epidermal growth factor receptor expression in normal and diseased human adrenal cortex by immunohistochemistry and in situ hybridization. *Mod Pathol* **7**, 741-746
232. Gicquel, C., Raffin-Sanson, M.-L., Gaston, V., Bertagna, X., Plouin, P.-F., Schlumberger, M., Louvel, A., Luton, J.-P., and Le Bouc, Y. (1997) Structural and Functional Abnormalities at 11p15 Are Associated with the Malignant Phenotype in Sporadic Adrenocortical Tumors: Study on a Series of 82 Tumors. *Journal of Clinical Endocrinology & Metabolism* **82**, 2559-2565
233. Voutilainen, R. (1998) Adrenocortical Cells are the Site of Secretion and Action of Insulin-Like Growth Factors and TNF-Alpha. *Horm Metab Res* **30**, 432,435
234. Bourcigaux, N., Gaston, V., Logié, A., Bertagna, X., Le Bouc, Y., and Gicquel, C. (2000) High Expression of Cyclin E and G1 CDK and Loss of Function of p57KIP2 Are Involved in Proliferation of Malignant Sporadic Adrenocortical Tumors. *Journal of Clinical Endocrinology & Metabolism* **85**, 322-330
235. McNicol, A. M., Nolan, C. E., Struthers, A. J., Farquharson, M. A., Hermans, J., and Haak, H. R. (1997) Expression of p53 in adrenocortical tumours: clinicopathological correlations. *The Journal of Pathology* **181**, 146-152
236. Reinke V, L. G. (1997) Differential activation of p53 targets in cells treated with ultraviolet radiation that undergo both apoptosis and growth arrest. *Radiat. Res.* **148**
237. Sameshima, Y., Tsunematsu, Y., Watanabe, S., Tsukamoto, T., Kawa-ha, K., Hirata, Y., Mizoguchi, H., Sugimura, T., Terada, M., and Yokota, J. (1992) Detection of Novel Germ-line p53 Mutations in Diverse-Cancer-Prone Families Identifiled by Selecting Patients With Childhood Adrencortical Carcinoma. *Journal of the National Cancer Institute* **84**, 703-707
238. Wagner, J., Portwine, C., Rabin, K., Leclerc, J.-M., Narod, S. A., and Malkin, D. (1994) High Frequency of Germline p53 Mutations in Childhood Adrenocortical Cancer. *Journal of the National Cancer Institute* **86**, 1707-1710
239. Tissier, F., Cavard, C., Groussin, L., Perlemoine, K., Fumey, G., Hagnere, A. M., Rene-Corail, F., Jullian, E., Gicquel, C., Bertagna, X., Vacher-Lavenu, M. C., Perret, C., and Bertherat, J. (2005) Mutations of {beta}-Catenin in Adrenocortical Tumors: Activation of the Wnt Signaling Pathway Is a

Frequent Event in both Benign and Malignant Adrenocortical Tumors. *Cancer Research* **65**, 7622-7627

240. Kjellman, M., Roshani, L., Teh, B. T., Kallioniemi, O.-P., Höög, A., Gray, S., Farnebo, L.-O., Holst, M., Bäckdahl, M., and Larsson, C. (1999) Genotyping of Adrenocortical Tumors: Very Frequent Deletions of the MEN1 Locus in 11q13 and of a 1-Centimorgan Region in 2p16. *Journal of Clinical Endocrinology & Metabolism* **84**, 730-735
241. Yano, T., Linehan, M., Angland, P., Lerman, M. I., Daniel, L. N., Stein, C. A., Roberston, C. N., LaRocca, R., and Zbar, B. (1989) Genetic Changes in Human Adrenocortical Carcinomas. *Journal of the National Cancer Institute* **81**, 518-519
242. Wales MM, B. M., el Deiry W, Nelkin BD, Issa JP, Cavenee WK, Kuerbitz SJ, Baylin SB (1995) P53 activates expression of HIC-1, a new candidate tumour suppressor gene on 17p13.3. *Nature Madicine* **1**, 570-577
243. Berruti A, T. M., Pia A, Angeli A, Dogliotti L (1998) Mitotane associated with etoposide, doxirubicin, and cisplatin in the treatment of advanced adrenocortical carcinoma. Italian Group for the Study of Adrenal Cancer. *Cancer* **83**, 2194-2200
244. Bonacci R, G. A., Baudin E (1998) Cytotoxic therapy with etoposide and cisplatin in advanced adrenocortical carcinoma. *J Cancer* **78**, 546-549
245. Bulun SE, N. L., Takayama K, Michael MD, Agarwal V, Fisher C, Zhao Y, Hinshelwood MM, Ito Y, Simpson ER. (1997) Endocrine disorders associated with inappropriately high aromatase expression. *J Steroid Biochem Mol Biol* **61**, 133-139
246. Phornphutkul, C., Okubo, T., Wu, K., Harel, Z., Tracy, T. F., Pinar, H., Chen, S., Gruppuso, P. A., and Goodwin, G. (2001) Aromatase P450 Expression in a Feminizing Adrenal Adenoma Presenting as Isosexual Precocious Puberty. *Journal of Clinical Endocrinology & Metabolism* **86**, 649-652
247. Young, J., Bulun, S. E., Agarwal, V., Couzinet, B., Mendelson, C. R., Simpson, E. R., and Schaison, G. (1996) Aromatase expression in a feminizing adrenocortical tumor. *Journal of Clinical Endocrinology & Metabolism* **81**, 3173-3176
248. Pezzi, V., Mathis, J. M., Rainey, W. E., and Carr, B. R. (2003) Profiling transcript levels for steroidogenic enzymes in fetal tissues. *The Journal of Steroid Biochemistry and Molecular Biology* **87**, 181-189
249. Baquedano, M. S., Saraco, N., Berensztein, E., Pepe, C., Bianchini, M., Levy, E., Goni, J., Rivarola, M. A., and Belgorosky, A. (2007) Identification and Developmental Changes of Aromatase and Estrogen Receptor Expression in Prepubertal and Pubertal Human Adrenal Tissues. *Journal of Clinical Endocrinology Metabolism* **92**, 2215-2222
250. Phornphutkul, C., Okubo, T., Wu, K., Harel, Z., Tracy, T. F., Jr., Pinar, H., Chen, S., Gruppuso, P. A., and Goodwin, G. (2001) Aromatase P450 Expression in a Feminizing Adrenal Adenoma Presenting as Isosexual Precocious Puberty. *Journal of Clinical Endocrinology Metabolism* **86**, 649-652
251. Moslemi S, V. A., Papadopoulos V, Camoin L, Silberzahn P,Gaillard JL (1997) Purification and characterization of equine testicular cytochrome P-

450 aromatase: comparison with the human enzyme. *Comp Biochem Physiol B Biochem Mol Biol* **118**, 217-227
252. Sethumadhavan K, B. F., Thotakura NR (1991) Estrogen synthetase (aromatase). The cytochrome P-450 component of the human placental enzyme is a glycoprotein. *Mol Cell Endocrinol* **78**, 25-32
253. Giorgetti, S., Ballotti, R., Kowalski-Chauvel, A., Tartare, S., and Van Obberghen, E. (1993) The insulin and insulin-like growth factor-I receptor substrate IRS-1 associates with and activates phosphatidylinositol 3-kinase in vitro. *Journal of Biological Chemistry* **268**, 7358-7364
254. Weber, M. M., Auernhammer, C. J., Kiess, W., and Engelhardt, D. (1997) Insulin-like growth factor receptors in normal and tumorous adult human adrenocortical glands. *European Journal of Endocrinology* **136**, 296-303
255. Haluska, P., Worden, F., Olmos, D., Yin, D., Schteingart, D., Batzel, G., Paccagnella, M., de Bono, J., Gualberto, A., and Hammer, G. (2010) Safety, tolerability, and pharmacokinetics of the anti-IGF-1R monoclonal antibody figitumumab in patients with refractory adrenocortical carcinoma. *Cancer Chemotherapy and Pharmacology* **65**, 765-773
256. Leary, A., and Dowsett, M. (2006) Combination therapy with aromatase inhibitors: the next era of breast cancer treatment? *Br J Cancer* **95**, 661-666
257. Lewis-Wambi JS, J. V. (2005) Treatment of Postmenopausal Breast Cancer with Selective Estrogen Receptor Modulators (SERMs). *Breast Dis* **24**, 93-105
258. Barnes CJ, V. R., Kumar R (2004) Novel estrogen receptor coregulators and signaling moleculesin human diseases. *Cell Mol. Life Sci.* **61**, 281-291
259. Wong, C.-W., McNally, C., Nickbarg, E., Komm, B. S., and Cheskis, B. J. (2002) Estrogen receptor-interacting protein that modulates its nongenomic activity-crosstalk with Src/Erk phosphorylation cascade. *Proceedings of the National Academy of Sciences* **99**, 14783-14788
260. Khan, M. M., Hadman, M., Wakade, C., De Sevilla, L. M., Dhandapani, K. M., Mahesh, V. B., Vadlamudi, R. K., and Brann, D. W. (2005) Cloning, Expression, and Localization of MNAR/PELP1 in Rodent Brain: Colocalization in Estrogen Receptor-α- But Not in Gonadotropin-Releasing Hormone-Positive Neurons. *Endocrinology* **146**, 5215-5227
261. Choi, Y. B., Ko, J. K. and Shin, J (2004) The transcriptional corepressor, PELP1, recruits HDAC2 and masks histones using two separate domains. *J Biol Chem* **279**, 50930-50941
262. Vadlamudi, R. K., Bagheri-Yarmand, R., Yang, Z., Balasenthil, S., Nguyen, D., Sahin, A. A., den Hollander, P. and Kumar, R (2004) Dynein light chain 1, a p21-activated kinase 1-interacting substrate, promotes cancerous phenotypes. *Cancer Cell* **5**, 575-585
263. Singh, R. R., Gururaj, A. E., Vadlamudi, R. K. and Kumar, R (2006) 9-cis-retinoic acid up-regulates expression of transcriptional coregulator PELP1, a novel coactivator of the retinoid X receptor α pathway. *J Biol Chem* **281**, 15394-15404
264. Rosendorff, A., Sakakibara, S., Lu, S., Kieff, E., Xuan, Y., DiBacco, A., Shi, Y., Shi, Y. and Gill, G (2006) NXP-2 association with SUMO-2 depends on lysines required for transcriptional repression. *Proc Natl Acad Sci U S A* **103**, 5308-5313

265. Nair, S. a. V., R. K. (2007) Emerging significance of ER-coregulator PELP1/MNAR in cancer. *Histol Histopathol* **22**, 91-96
266. Balasenthil, S. a. V., R. K. (2003) Functional interactions between the estrogen receptor coactivator PELP1/MNAR and retinoblastoma protein. *J Biol Chem* **278**, 22119-22127
267. Haas, D., White, S. N., Lutz, L. B., Rasar, M. and Hammes, S. R. (2005) The modulator of nongenomic actions of the estrogen receptor (MNAR) regulates transcription-independent androgen receptor-mediated signaling: evidence that MNAR participates in G protein-regulated meiosis in Xenopus laevis oocytes. *Mol Endocrinol* **19**, 2035-2046
268. Vadlamudi, R. K., Balasenthil, S., Sahin, A. A., Kies, M., Weber, R. S., Kumar, R. and El-Naggar, A. K (2005) Novel estrogen receptor coactivator PELP1/MNAR gene and ERbeta expression in salivary duct adenocarcinoma: potential therapeutic targets. *Hum Pathol* **36**, 670-675
269. Rajhans, R. a. V., R. K. (2006) Comprehensive analysis of recent biochemical and biologic findings regarding a newly discovered protein-PELP1/MNAR. *Clin Exp Metastasis* **23**, 1-7
270. Greger, J. G., Guo, Y., Henderson, R., Ross, J. F. and Cheskis, B. J. (2006) Characterization of MNAR expression. *Steroids* **71**, 317-322
271. Pawlak, J. a. B., C. (2005) Developmental expression of MNAR mRNA in the mouse brain. *Cell Tissue Res* **320**, 545-549
272. Mishra, S. K., Balasenthil, S., Nguyen, D. and Vadlamudi, R. K. (2004) Cloning and functional characterization of PELP1/MNAR promoter. *Gene* **330**, 115-122
273. Nagpal, J. K., Nair, S., Chakravarty, D., Rajhans, R., Pothana, S., Brann, D. W., Tekmal, R. R., and Vadlamudi, R. K. (2008) Growth Factor Regulation of Estrogen Receptor Coregulator PELP1 Functions via Protein Kinase A Pathway. *Molecular Cancer Research* **6**, 851-861
274. Ballif, B. A., Villen, J., Beausoleil, S. A., Schwartz, D. and Gygi, S. P. (2004) Phosphoproteomic analysis of the developing mouse brain. *Mol Cell Proteomics* **10**, 1093-1101
275. Mishra S. K., M. A., Vadlamudi R. K., Li F., Wang R. A., Yu W., Jordan V. C., Santen R. J., Kumar R. (2003) MICoA, a novel metastasis-associated protein 1 (MTA1) interacting protein coactivator, regulates estrogen receptor-α transactivation functions. *J Biol Chem.* **278**, 19209-19219
276. Joung, I., Strominger, J. L., and Shin, J. (1996) Molecular cloning of a phosphotyrosine-independent ligand of the p56lck SH2 domain. *Proc Natl Acad Sci U S A* **93**, 5991-5995
277. Rayala, S. K., Hollander, P., Balasenthil, S., Molli, P. R., Bean, A. J., Vadlamudi, R. K., Wang, R. A., and Kumar, R. (2006) Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) interacts with PELP1 and activates MAPK. *J Biol Chem.* **281**, 4395-4403
278. Vadlamudi, R. K., and Kumar, R. (2007) Functional and biological properties of the nuclear receptor coregulator PELP1/MNAR *Nuclear Receptor Signaling* **5**, e004
279. Popov, V. M., Zhou, J., Shirley, L. A., Quong, J., Yeow, W. S., Wright, J. A., Wu, K., Rui, H., Vadlamudi, R. K., Jiang, J., Kumar, R., Wang, C., and Pestell, R. G. (2009) The Cell Fate Determination Factor DACH1 Is

Expressed in Estrogen Receptor-{alpha}-Positive Breast Cancer and Represses Estrogen Receptor-{alpha} Signaling. *Cancer Research* **69**, 5752-5760

280. Grivas, P., Tzelepi, V., Sotiropoulou-Bonikou, G., Kefalopoulou, Z., Papavassiliou, A., and Kalofonos, H. (2009) Expression of ERalpha, ERbeta and co-regulator PELP1/MNAR in colorectal cancer: prognostic significance and clinicopathologic correlations. *Cell Oncol* **31**, 235-247
281. Tzelepi V, G. P., Kefalopoulou Z, Kalofonos H, Varakis JN, Sotiropoulou-Bonikou G. (2009) Expression of estrogen receptor co-regulators NCoR and PELP1 in epithelial cells and myofibroblasts of colorectal carcinomas: cytoplasmic translocation of NCoR in epithelial cells correlates with better [corrected] prognosis. *Virchows Arch.* **454**, 41-53
282. Márquez-Garbán, D., Chen, H., Fishbein, M., Goodglick, L., and RJ., P. (2007) Estrogen receptor signaling pathways in human non-small cell lung cancer. *Steroids* **72**
283. Gururaj, A. E., Rayala, S. K., Vadlamudi, R. K., and Kumar, R. (2006) Novel Mechanisms of Resistance to Endocrine Therapy: Genomic and Nongenomic Considerations. *Clinical Cancer Research* **12**, 1001s-1007s
284. Mishra, S. K., Talukder, A. H., Gururaj, A. E., Yang, Z., Singh, R. R., Mahoney, M. G., Francí, C., Vadlamudi, R. K., and Kumar, R. (2004) Upstream Determinants of Estrogen Receptor-α Regulation of Metastatic Tumor Antigen 3 Pathway. *Journal of Biological Chemistry* **279**, 32709-32715
285. Rainey, W. E., Bird, I. M., and Mason, J. I. (1994) The NCI-H295 cell line: a pluripotent model for human adrenocortical studies. *Molecular and Cellular Endocrinology* **100**, 45-50
286. Weiss, L. M., Medeiros, L. J., and Vickery, A. L., Jr. (1989) Pathologic features of prognostic significance in adrenocortical carcinoma. *Am J Surg Pathol* **13**, 202-206
287. Aubert, S. b., Wacrenier, A., Leroy, X., Devos, P., Carnaille, B., Proye, C., Wemeau, J. L., Lecomte-Houcke, M., and Leteurtre, E. (2002) Weiss System Revisited: A Clinicopathologic and Immunohistochemical Study of 49 Adrenocortical Tumors. *The American Journal of Surgical Pathology* **26**
288. Genua, M., Pandini, G., Sisci, D., Castoria, G., Maggiolini, M., Vigneri, R., and Belfiore, A. (2009) Role of Cyclic AMP Response Element-Binding Protein in Insulin-like Growth Factor-I Receptor Up-regulation by Sex Steroids in Prostate Cancer Cells. *Cancer Research* **69**, 7270-7277
289. Cascio, S., Bartella, V., Garofalo, C., Russo, A., Giordano, A., and Surmacz, E. (2007) Insulin-like Growth Factor 1 Differentially Regulates Estrogen Receptor-dependent Transcription at Estrogen Response Element and AP-1 Sites in Breast Cancer Cells. *Journal of Biological Chemistry* **282**, 3498-3506
290. Castro-Rivera, E., Samudio, I., and Safe, S. (2001) Estrogen Regulation of Cyclin D1 Gene Expression in ZR-75 Breast Cancer Cells Involves Multiple Enhancer Elements. *Journal of Biological Chemistry* **276**, 30853-30861
291. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon, P. (1995) Activation of the estrogen receptor through

phosphorylation by mitogen-activated protein kinase. *Science.* **270**, 1491-1494

292. Lee, A. V., Weng, C. N., Jackson, J. G., and Yee, D. (1997) Activation of estrogen receptor-mediated gene transcription by IGF-I in human breast cancer cells. *Journal of Endocrinology* **152**, 39-47
293. Chakravarty, D., Tekmal, R. R., and Vadlamudi, R. K. (2010) PELP1: A novel therapeutic target for hormonal cancers. *IUBMB Life* **62**, 162-169
294. Rajhans, R., Nair, H. B., Nair, S. S., Cortez, V., Ikuko, K., Kirma, N. B., Zhou, D., Holden, A. E., Brann, D. W., Chen, S., Tekmal, R. R., and Vadlamudi, R. K. (2008) Modulation of in Situ Estrogen Synthesis by Proline-, Glutamic Acid-, and Leucine-Rich Protein-1: Potential Estrogen Receptor Autocrine Signaling Loop in Breast Cancer Cells. *Molecular Endocrinology* **22**, 649-664
295. Terzolo, M., Angeli, A., Fassnacht, M., Daffara, F., Tauchmanova, L., Conton, P. A., Rossetto, R., Buci, L., Sperone, P., Grossrubatscher, E., Reimondo, G., Bollito, E., Papotti, M., Saeger, W., Hahner, S., Koschker, A. C., Arvat, E., Ambrosi, B., Loli, P., Lombardi, G., Mannelli, M., Bruzzi, P., Mantero, F., Allolio, B., Dogliotti, L., and Berruti, A. (2007) Adjuvant Mitotane Treatment for Adrenocortical Carcinoma. *The New England Journal of Medicine* **356**, 2372-2380
296. Sbiera, S., Schmull, S., Assie, G., Voelker, H. U., Kraus, L., Beyer, M., Ragazzon, B., Beuschlein, F., Willenberg, H. S., Hahner, S., Saeger, W., Bertherat, J., Allolio, B., and Fassnacht, M. (2010) High Diagnostic and Prognostic Value of Steroidogenic Factor-1 Expression in Adrenal Tumors. *Journal of Clinical Endocrinology Metabolism* **95**, E161-E171
297. Katzenellenbogen, B. S., and Norman, M. J. (1990) Multihormonal regulation of the progesterone receptor in MCF-7 human breast cancer cells: interrelationships among insulin/insulin-like growth factor-I, serum, and estrogen. *Endocrinology* **126**, 891-898
298. Varma, H., and Conrad, S. E. (2002) Antiestrogen ICI 182,780 Decreases Proliferation of Insulin-like Growth Factor I (IGF-I)-treated MCF-7 Cells without Inhibiting IGF-I Signaling. *Cancer Research* **62**, 3985-3991
299. Pandini, G., Genua, M., Frasca, F., Squatrito, S., Vigneri, R., and Belfiore, A. (2007) 17β-Estradiol Up-regulates the Insulin-like Growth Factor Receptor through a Nongenotropic Pathway in Prostate Cancer Cells. *Cancer Research* **67**, 8932-8941
300. Simoncini, T., Hafezi-Moghadam, A., Brazil, D. P., Ley, K., Chin, W. W., and Liao, J. K. (2000) Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* **407**, 538-541
301. Sun, M., Paciga, J. E., Feldman, R. I., Yuan, Z. q., Coppola, D., Lu, Y. Y., Shelley, S. A., Nicosia, S. V., and Cheng, J. Q. (2001) Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, Activated in Breast Cancer, Regulates and Is Induced by Estrogen Receptor à (ERà) via Interaction between ERà and PI3K. *Cancer Research* **61**, 5985-5991
302. Razandi, M., Oh, P., Pedram, A., Schnitzer, J., and Levin, E. R. (2002) ERs Associate with and Regulate the Production of Caveolin: Implications for Signaling and Cellular Actions. *Molecular Endocrinology* **16**, 100-115

303. Weinstein, I. B. (1996) Relevance of cyclin D1 and other molecular markers to cancer chemoprevention. *J Cell Biochem Suppl.* **25**, 23-28
304. Dufourny, B., van Teeffelen, H. A., Hamelers, I. H., Sussenbach, J. S., and Steenbergh, P. H. (2000) Stabilization of cyclin D1 mRNA via the phosphatidylinositol 3-kinase pathway in MCF-7 human breast cancer cells. *Journal of Endocrinology* **166**, 329-338
305. Zhang, S., Li, X., Burghardt, R., Smith, R., III, and Safe, S. H. (2005) Role of estrogen receptor (ER) {alpha} in insulin-like growth factor (IGF)-I-induced responses in MCF-7 breast cancer cells. *Journal of Molecular Endocrinology* **35**, 433-447
306. Butt, A. J., McNeil, C. M., Musgrove, E. A., and Sutherland, R. L. (2005) Downstream targets of growth factor and oestrogen signalling and endocrine resistance: the potential roles of c-Myc, cyclin D1 and cyclin E. *Endocrine-Related Cancer* **12**, S47-S59
307. Surmacz, E. (2003) Growth factor receptors as therapeutic targets: strategies to inhibit the insulin-like growth factor I receptor. *Oncogene* **22**, 6589-6597
308. Klotz, D. M., Hewitt, S. C., Ciana, P., Raviscioni, M., Lindzey, J. K., Foley, J., Maggi, A., DiAugustine, R. P., and Korach, K. S. (2002) Requirement of Estrogen Receptor à in Insulin-like Growth Factor-1 (IGF-1)-induced Uterine Responses and in Vivo Evidence for IGF-1/Estrogen Receptor Cross-talk. *Journal of Biological Chemistry* **277**, 8531-8537
309. Nair, B. C., Nair, S., Chakravarty, D., Rajhans, R., Cortez, V., Tekmal, R. R., and Vadlamudi, R. K. (2008) PELP1/MNAR: a novel CDKs substrate and regulator of pRb/E2F pathway. *Proc. Am. Assn. Cancer Res.* **4222.**
310. Young, M., and McPhaul, M. J. (1997) Definition of the elements required for the activity of the rat aromatase promoter in steroidogenic cell lines. *J Steroid Biochem Mol Biol* **61**, 341-348
311. Lonard, D., and BW, O. M. (2006) The expanding cosmos of nuclear receptor coactivators. *Cell Mol. Life Sci.* **125**, 411-414
312. Smith, C., and O'Malley, B. (2004) Coregulator function: a key to understanding tissue specificity of selective receptor modulators. *Endocrine Reviews* **25**, 45-71
313. Stein, R., and McDonnell, D. (2006) Estrogen-related receptor as a therapeutic target in cancer. *Endocr Relat Cancer* **13**, S25-S32
314. Chen, S., Itoh, T., Wu, K., Zhou, D., and Yang, C. (2002) Transcriptional regulation of aromatase expression in human breast tissue. *J Steroid Biochem Mol Biol* **83**, 93-99
315. Unni, E., Sun, S., Nan, B., Mcphaul, M. J., Cheskis, B., Mancini, M. A., and Marcelli, M. (2004) Changes in androgen receptor nongenotropic signaling correlate with transition of LNCaP cells to androgen independence. *Cancer Res.* **64**, 7156-7168
316. Kayahara, M., Ohanian, J., Ohanian, V., Berry, A., Vadlamudi, R., and Ray, D. W. (2008) MNAR functionally interacts with both NH2- and COOH-terminal GR domains to modulate transactivation. *J. Physiol. Endocrinol. Metab.* **295**, E1047–E1055
317. Khan, M. M., Hadman, M., De Sevilla, L. M., Mahesh, V. B., Buccafusco, J., Hill, W. D., and Brann, D. W. (2006) Cloning, distribution and colocalization

of MNAR/PELP1 with glucocorticoid receptors in primate and nonprimate brain. *Neuroendocrinology* **84**, 317–329

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Inhibition of Cyclooxygenase-2 Down-regulates Aromatase Activity and Decreases Proliferation of Leydig Tumor Cells*

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Our recent studies have revealed that estrogens stimulate an autocrine mechanism determining Leydig tumor cell proliferation. Estrogen overproduction is due to an elevated steroidogenic factor-1 (SF-1) expression and cAMP-response element-binding protein (CREB) phosphorylation, both inducing aromatase overexpression. Although we have shown that increased SF-1 expression depends mainly on higher local insulin-like growth factor I production, the mechanisms and factors determining increased CREB activation in Leydig tumor cells are not completely understood. In this study, we investigated the role of cyclooxygenase-2 (COX-2) in CREB dependent-aromatase expression in Leydig tumor cells. We found that COX-2 is expressed in rat and human Leydigiomas as well as in the rat Leydig tumor cell line R2C, but not in normal testis. Our data indicate that in R2C cells the COX-2-derived prostaglandin E2 (PGE2) binds the PGE2 receptor EP4 and activates protein kinase A (PKA) and ultimately CREB. Inhibitors for COX-2 (NS398), EP4 (AH23848), and PKA (H89) decreased aromatase expression and activity as a consequence of a decreased phosphorylated CREB recruitment to the PII promoter of the aromatase gene. The COX-2/PGE2/PKA pathway also seems to be involved in aromatase post-translational activation, an observation that requires further studies. The reduction in aromatase activity was responsible for a drop in estrogen production and subsequent reduction in cyclin E expression resulting in a decrease in tumor Leydig cell proliferation. Furthermore, COX-2 silencing caused a significant decrease in CREB phosphorylation, aromatase expression, and R2C cell proliferation. These novel findings clarify the mechanisms involved in the growth of Leydig cell tumors and should be taken into account in determining new therapeutic approaches.

Leydig cell tumors are the most common cancers of the gonadal stroma (1). However, their etiology and pathogenesis are poorly defined. Transgenic mice overexpressing aromatase, the enzyme responsible for the conversion of androgens to estrogens (2), present enhancement of circulating 17β-estradiol (E2)[3] levels, which have been associated with Leydig cell hyperplasia and tumors (3). These observations and our recent data (4, 5) suggest that estrogens can elicit proliferative effects in human and rat tumor Leydig cells through an autocrine mechanism.

Aromatase activity is regulated primarily at the level of gene expression and is present in testicular somatic cells and during the maturative phases of male germ cells (6). The *CYP19* gene that encodes aromatase contains at least eight unique promoters used in a tissue-specific manner (2). The proximal promoter II regulates aromatase expression in human fetal and adult testes, R2C, and H540 rat Leydig tumor cells (7). Specific response elements appear to be involved in rat aromatase expression: a nuclear receptor half site binding SF-1/LRH-1 (8) and CRE-like sequences binding CREB/ATF protein family members (9, 10). In a recent study, we have shown that Leydig cell tumors exhibit increased SF-1 expression and CREB phosphorylation, which is an indicator of elevated transcriptional activity (4). In the same study, we explained that Leydig tumor cells produce high levels of insulin-like growth factor I, which, through the activation of phosphatidylinositol 3-kinase and Akt pathways, leads to increased steroidogenic factor-1 (SF-1) transcription. Moreover, R2C cells are characterized by the absence of DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome, gene *1*), an SF-1 corepressor. This is probably due to constitutively active PKA signaling, because in a mouse Leydig cell line, a marked decrease of DAX-1 mRNA was observed after addition of LH (luteinizing hormone) and forskolin (11). Increased SF-1 binding to promoter PII, together with pCREB, contribute to constitutive active aromatase expression, which, in turn, is responsible for E2 production involved in Leydig tumor cell proliferation. Although we have elucidated the pathway responsible for increased SF-1 transcription, the molecular mechanisms determining constitutive CREB phosphorylation still remain to be defined.

It has been shown that prostaglandin E2 (PGE2) stimulates cAMP formation and production of promoter II-specific aromatase transcripts in human adipose tissue (12). The first step in prostaglandin (PG) synthesis from arachidonic acid is catalyzed by two isoforms of cyclooxygenase (COX),

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[3] The abbreviations used are: E2, 17β-estradiol; COX-2, cycloxygenase-2; CREB, cAMP-responsive binding protein; pCREB, phosphorylated CREB; SF-1, steroidogenic factor-1; MAP, mitogen-activated protein; ERK1/2, extracellular regulated kinase 1/2; PGE2, prostaglandin E2; EP, E-series prostanoid receptors; CYP, cytochrome P450; FRNT, Fischer rat normal testis; FRTT, Fischer rat tumor testis; ChIP, chromatin immunoprecipitation; DAX-1, dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome gene *1*; LH, luteinizing hormone; LHR, LH receptor; siRNA, small interference RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription.

which are the rate-limiting enzymes in PG synthesis because of their rapid auto-inactivation (13). COX-1 is constitutively present in various tissues and regulates the physiological production of PGs necessary for maintaining normal homeostasis. Conversely COX-2 represents the inducible isoform, and it is not detectable in most normal tissues but is rapidly induced by mitogens, cytokines, and growth factors (reviewed in Refs. 14, 15).

Recently, overexpression of COX-2 has been observed in several tumor types such as colorectal (16), gastric (17), hepatocellular (18), lung (19), esophageal (20), and pancreatic carcinoma (21). COX-2 was also detected in breast cancer (22), where it was associated with aromatase expression (23). It is worth noting that COX inhibitors decreased both aromatase expression and activity in breast cancer cells (24), and the use of combinations of COX-2 and aromatase inhibitors was more effective than single agents in decreasing estradiol production (25).

COX-2 was not present in normal human testes, but was found in testicular biopsies from men with impaired spermatogenesis (26) or in testicular cancer (27). On the basis of the aforementioned observations, in the present study we evaluated the potential stimulatory action exerted by COX-2-derived PGE2, on aromatase expression, E2 production, and proliferation of Leydig tumor cells. We used, as model systems, rat R2C Leydig cancer cells and Leydig cell tumors from Fisher rats, which were characterized by exceptionally high incidence of spontaneous neoplasms associated with aging (28). Here, we demonstrate that the COX-2/PGE2/PKA transduction pathway is directly involved in CREB phosphorylation, which, in turn, stimulates aromatase expression and E2-dependent Leydig tumor cell proliferation.

EXPERIMENTAL PROCEDURES

Cell Cultures, Animals, and Reagents—Cells were obtained from ATCC (LGC Standards, Teddington, Middlesex, UK), grown for 2 weeks (four passages) before freezing aliquots. Each aliquot was used for no more than ten passages. TM3 cells (an immature mouse Leydig cell line) were cultured in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 5% horse serum, 2.5% fetal bovine serum, and antibiotics (Invitrogen); R2C cells (a rat Leydig tumor cell line) were cultured in Ham's nutrient mixture F-10 supplemented with 15% horse serum, 2.5% fetal bovine serum, and antibiotics (Invitrogen). Six-month-old male Fischer rat normal (FRN) and 24-month-old male Fischer rat with a tumor (FRT) (a generous gift of Sigma-Tau Pomezia, Italy) were used for the studies. At 24 months the animals spontaneously developed Leydig cell tumors; these tumors 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. NS398, AH23848 (29), AH6809 (30), SC19220 (31–33), and H89 (Sigma) were used.

Immunohistochemical Analysis—Analysis was performed on formalin-fixed and paraffin-embedded testis tissues from 2 male patients with Leydig cell tumor (32 and 33 years of age) and 2 male patients (30 and 35 years of age) with a testicular granulomatous-like lesion (control tissue). The archival cases were provided by the Pathologic Anatomy Unit (Annunziata Hospital, Cosenza, Italy). Tissues were heated for antigen retrieval. Paraffin-embedded sections, 5 μm thick, were mounted on slides precoated with polylysine, and then deparafinized and dehydrated (7–8 serial sections). Hydrogen peroxide (3% in distilled water) was used for 30 min to inhibit endogenous peroxidase activity while normal horse serum (10%) was utilized for 30 min to block nonspecific binding sites. Immunodetection was carried out using anti-COX-2 (1:50), primary antibodies at 4 °C overnight. A biotinylated universal horse IgG was applied (1:600) for 1 h at room temperature, followed by the avidin-biotin-horseradish peroxidase complex (Vector, Laboratories, CA). Immunoreactivity was visualized by using the diaminobenzidine chromogen (Zymed Laboratories Inc.). Sections were also counterstained with hematoxylin. The primary antibody was replaced by normal rabbit serum in negative control sections.

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]androst-4-ene-3,17-dione (PerkinElmer Life Sciences) as a substrate (34). Incubations were performed at 37 °C for 2 h under a 95%:5% air/CO_2 atmosphere. Results obtained were expressed as picomoles/h and normalized to milligrams of protein (picomoles/h/mg of protein).

Radioimmunoassay—Prior to experiments, TM3 cells were maintained overnight in Dulbecco's modified Eagle's medium/F-12 medium and R2C cells in Ham/F-10 without serum or antibiotics (serum-free medium). Cells were then treated as indicated, and the estradiol content of medium recovered from each well was determined against standards prepared in serum-free medium using a radioimmunoassay kit (Diagnostic System Laboratories, Webster, TX). Results were normalized to the cellular protein content per well.

Real-time RT-PCR—Prior to experiments, cells were maintained overnight in serum-free 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) Fischer 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 prior to 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). The 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 rat *CYP19*. The nucleotide sequences of the primers for *CYP19* were as follows: forward, 5′-GAGAAACTGGAAGACTGTATGGAT-3′ and reverse, 5′-ACTGATTCACGTTCTCCTTTGTCA-3′. PCR reactions were performed in the iCycler iQ Detection System (Bio-Rad), using 0.1 μM of each primer, in a total volume of 30 μl of 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 18 S ribosomal RNA content. The 18 S quantification was performed using a TaqMan Ribosomal RNA Reagent kit (Applied Biosystems, Applera Italia, Monza, Milano, Italy) following the method provided in the TaqMan Ribosomal RNA Control Reagent Kit. 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 18 S rRNA and calibrator, calculated following the $\Delta\Delta C_t$ method, as published previously (4).

Chromatin Immunoprecipitation (ChIP) Assay—This assay was performed using the ChIP Assay Kit from Upstate (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 NS398 and H89 (Sigma). 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) 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. Of the supernatants 10 μl was kept as input (starting material, to normalize results), whereas 100 μl was diluted 1:10 in 900 μl of ChIP dilution buffer (Upstate) and immunocleared with 80 μl of sonicated salmon sperm DNA protein A-agarose (Upstate) for 6 h at 4 °C. The immunocomplex was formed 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 for 4 h. DNA-protein complexes were reverse cross-linked overnight at 65 °C. Extracted DNA was resuspended in 20 μl of buffer containing 10 mM Tris and 1 mM EDTA, pH 8.0. A 5-μl volume of each sample and input were used for real-time PCR using *CYP19* promoter II-specific primers: forward, 5′-TCAAGGGTAGGAATTGGGA-C-3′; reverse, 5′-GGTGCTGGAATGGACAGATG-3′. PCR reactions were performed in the iCycler iQ Detection System (Bio-Rad), 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 (Bio-Rad), with the dissociation protocol, was used for gene amplification. Negative controls contained water instead of DNA. Final results were calculated using the $\Delta\Delta C_t$ method as explained above, using input C_t values instead of the 18 S. The basal sample was used as calibrator.

PGE2 Production—TM3 and R2C cells were maintained in complete medium for 48 h and in serum-free medium overnight. The following day fresh serum-free medium or, in the case of R2C cells, medium with NS398 was added for 6 h. PGE2 in the culture medium was assayed using an EIA kit following the manufacturer's instructions (Cayman).

Western Blot Analysis—Methods for protein extraction and blot preparation have been previously published (4). Blots were incubated overnight at 4 °C with (*a*) anti-COX-2 (1:1000, Cell Signaling Technology), (*b*) anti- COX-1 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), (*c*) anti-human P450 aromatase (1:50, Serotec, Oxford, UK), (*d*) anti-CREB (1:1000, 48H2, Cell Signaling Technology), (*e*) anti-pCREB Ser-133 (87G3, 1:1000, Cell Signaling Technology), (*f*) anti-cyclin E (M-20, 1:1000, Santa Cruz Biotechnology), (*g*) anti-EP4 (1:200, Santa Cruz Biotechnology), and (*h*) anti-GAPDH (1:1000, Santa Cruz Biotechnology). Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences), and immunoreactive bands were visualized with the ECL Western blotting detection system (Amersham Biosciences). To assure equal loading of proteins, membranes were stripped and incubated overnight with GAPDH antibody.

In Vitro Assay for PKA Activity—This assay was performed using the PepTag for Non-Radioactive Detection of cAMP-dependent Protein Kinase Assay kit (Promega). R2C cells were grown in 100 × 60-mm plates to 100% confluence. After treatments (1 h) cells were washed with phosphate-buffered saline (5 ml/100-mm dish) and lysed in cold PKA extraction buffer (0.5 ml/plate) containing 25 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 1 μg/ml leupeptin, 1 μg/ml aprotinin.

The lysates were cleared by centrifugation at 14,000 × *g*, 5 min, and 5 μl of cleared lysates was subjected to a kinase reaction with the fluorescence-labeled PKA substrate, Leu-Arg-Arg-Ala-Ser-Leu-Gly (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 100 volts for 15 min. 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 and EP4 stealth siRNA and scrambled siRNA were purchased from Invitrogen. Forty-eight hours after plating cells into 100-mm dishes at 7×10^6 cells, siRNAs were transfected in Ham/F-10 to a final concentration of 100 nM using the Lipofectamine 2000 Transfection Reagent (Invitrogen) according to manufacturer's instructions. Forty-eight hours after transfection cells were lysed for protein extraction. COX-2- and EP4-specific knockdown and effects on aromatase were analyzed by Western analysis.

Cell Proliferation Assay—For proliferative analysis a total of 1×10^5 cells was seeded onto 12-well plates in complete medium and grown for 2 days. Prior to experiments, cells were maintained for 24 h in Ham/F-10 medium and then treated with NS398, AH23848, and H89 (Sigma) or transfected with COX-2 siRNA (Invitrogen) as described above. 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 (PerkinElmer 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 M NaOH at 37 °C for 30 min. The total suspension was added to 10 ml of Optifluor fluid and was counted in a scintillation counter.

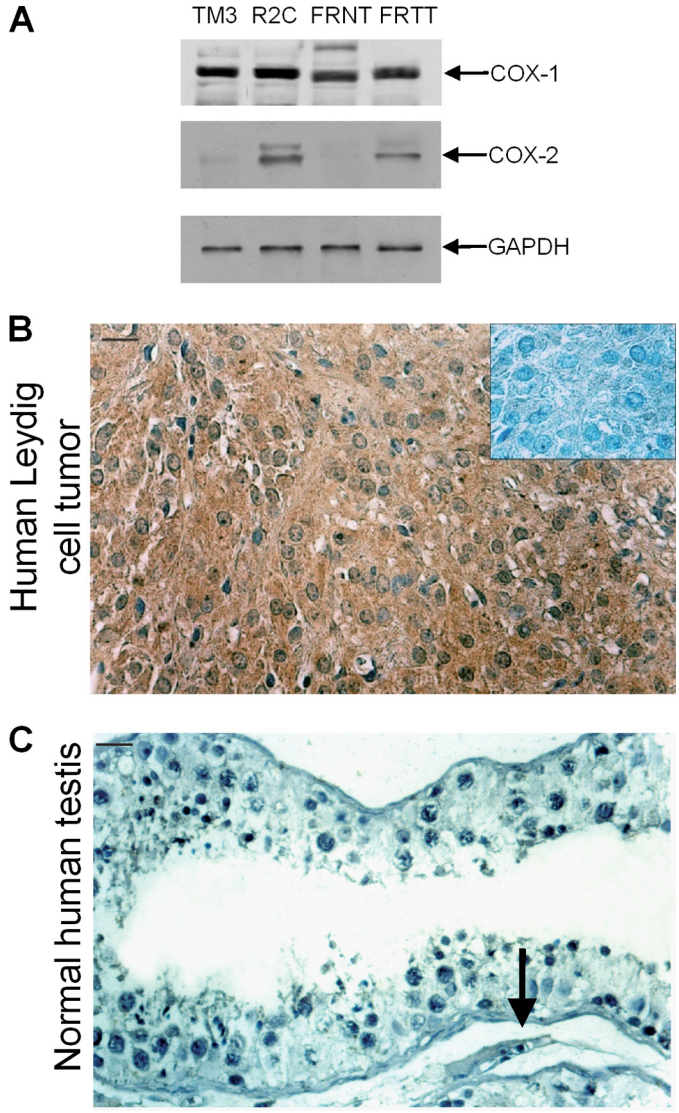

FIGURE 1. **Expression of COX-2 in Leydig tumor cells.** *A*, Western blot analysis was performed using 50 μg of total proteins extracted from TM3 and R2C cells or from total tissue of normal (FRNT) and tumor (FRTT) Fischer rat testes. GAPDH was used as a loading control. Results are representative of three independent experiments. *B* and *C*, immunodetection of COX-2 in Leydig cell tumor (*B*) and normal human testis (*C*). *Inset*: negative control. *Arrow*: Leydig cell. *Scale bars* = 12.5 μm.

Data Analysis and Statistical Methods—Pooled results from triplicate experiments were analyzed using one-way analysis of variance with Student-Newman-Keuls multiple comparison methods, using SigmaStat version 3.0 (SPSS, Chicago, IL).

RESULTS

COX-2 Is Highly Expressed in Leydig Cell Tumors—Using Western analysis we investigated COX-2 expression in normal and Leydig tumor cells (Fig. 1*A*). Both R2C cells in basal condition as well as testes from Fischer rats with a developed tumor (FRTT) express COX-2, which is absent in normal controls. On the other hand, COX-1 appears to be expressed at similar levels in all the samples (Fig. 1*A*). Moreover, by immunohistochemistry, we found similar results in human samples; the cytoplasm of neoplastic human Leydig cells showed a strong COX-2 immunoreactivity (Fig. 1*B*), while human control testis showed immunonegative reaction for COX-2 (Fig. 1*C*).

COX-2 Silencing and COX-2 Inhibitor, NS398, Decrease pCREB and Aromatase Expression—We have previously shown that expression of aromatase, SF-1, and the phosphorylated form of CREB are elevated in R2C and in rat Leydig tumor cells (4). The altered activation of CREB, together with high SF-1 expression, may explain the increase in aromatase levels observed in tumor Leydig cells. Knockdown of COX-2 in R2C cells using siRNA caused a significant decrease in aromatase expression together with a decrease in CREB phosphorylation (Fig. 2*A*). We also measured aromatase activity and found that the presence of COX-2 siRNA resulted in a 60% decrease in enzyme activity (Fig. 2*B*); similarly aromatase mRNA levels were decreased by 70% (Fig. 2*C*). Western blot analyses, using a specific COX-2 inhibitor NS398 (NS), showed that addition of increasing doses of NS (5, 25, and 50 μM) decreased both phosphorylated CREB and aromatase levels without affecting CREB expression (Fig. 3*A*). A drop in aromatase expression was also reflected by a change in enzymatic activity, which was dramatically reduced at all investigated NS doses (Fig. 3*B*). CREB is a transcription factor necessary for aromatase transcription via the PII promoter. As a consequence, the observed changes in pCREB levels induced changes in aromatase mRNA expression. As shown by real-time RT-PCR, the addition of different doses of NS resulted in a decrease in aromatase mRNA (Fig. 3*C*) without affecting CREB mRNA levels (data not shown), indicating a specific effect of COX-2 inhibition on CREB phosphorylation and pCREB binding to the aromatase PII promoter. We confirmed this hypothesis using a ChIP assay, which showed a specific decrease in pCREB binding (Fig. 3*D*, *left panel*) without any change in the amount of total CREB protein present on the aromatase PII promoter (Fig. 3*D*, *right panel*).

PGE2-activated Pathway Regulates Aromatase Expression—We first measured the ability of R2C cells to produce PGE2. We found that tumor cells were able to produce 650 ± 47 pg of PGE2/mg of protein (mean ± S.E. from three independent experiments; data not shown), an amount that is 5-fold higher than levels produced by TM3 cells. PGE2 levels in R2C cells were decreased by 60% in the presence of 50 μM NS398 (data not shown). Considering the high endogenous production of PGE2, only the addition of relatively high exogenous amounts of PGE2 to R2C cells were able to increase aromatase expression and CREB phosphorylation (Fig. 4*A*). When COX-2 was silenced, induction of aromatase expression was observed with 100-fold lower concentration of PGE2 (Fig. 4*B*), confirming that this prostaglandin is involved in aromatase regulation in R2C cells. Because PGE2 binds four different receptor isoforms, named EP (prostaglandin E receptor), we tested the effect of selective inhibitors for the different isoforms on aromatase and pCREB levels (Fig. 4*C*). Only the selective antagonist for the EP4 isoform, AH23848, resulted in a decrease in pCREB and in aromatase levels as well as a dose-dependent inhibition in aromatase activity (Fig. 4*D*). We then evaluated expression of the EP4 receptor isoform and found higher expression in R2C cells as well as in testes from Fischer rats with a developed tumor (FRTT) compared with the normal controls (Fig. 4*E*). To con-

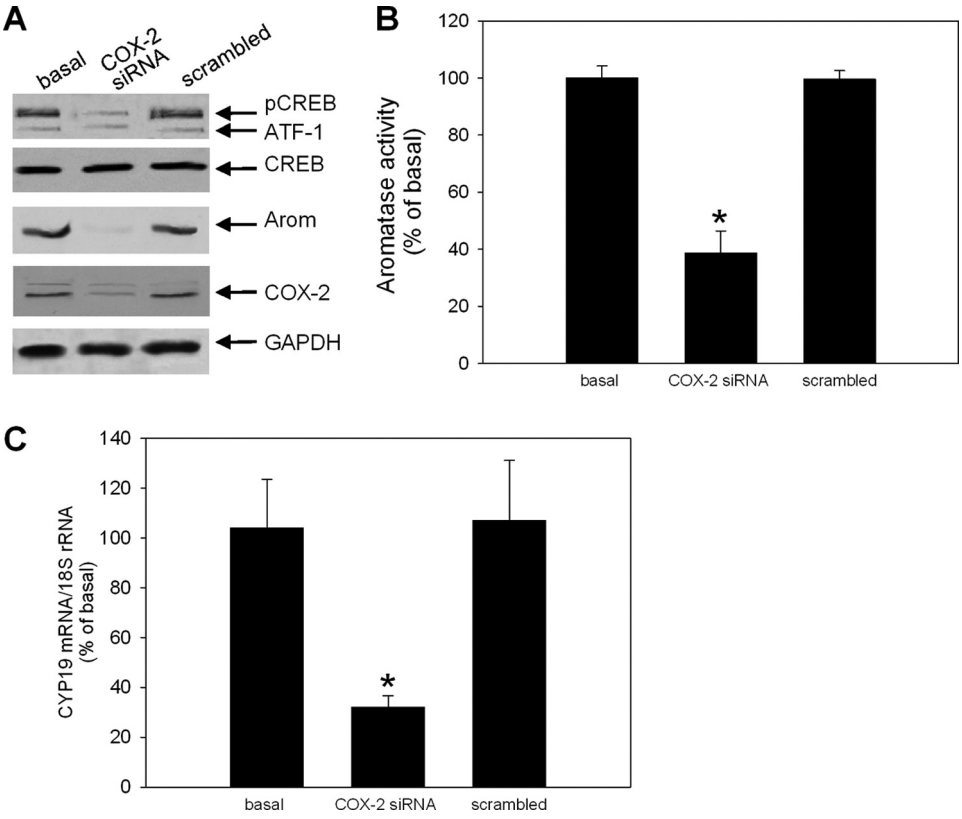

FIGURE 2. **Effects of COX-2 silencing on CREB phosphorylation and aromatase expression in R2C cells.** R2C cells were grown for 2 days in the presence of siRNA for COX-2 (100 nM) or scrambled siRNA (100 nM). *A*, Western blot analysis for aromatase (*Arom*), pCREB, CREB, and COX-2 was performed on 50 μg of total proteins extracted from R2C cells following siRNA gene silencing. GAPDH was used as a loading control. Results are representative of three independent experiments. *B*, aromatase activity was assessed using the modified tritiated water method. Results obtained were calculated as picomoles of $[^3H]H_2O$ released per hour normalized to the protein content per well (picomoles/h/mg of protein) and expressed as percent of basal. *Columns*, mean of three independent experiments each performed with triplicate samples expressed as percent of basal; *bars*, ±S.E. *C*, total RNA was extracted, and real-time RT-PCR was used to analyze mRNA levels of *CYP19*. Data represent the mean ± S.E. of values from three separate RNA samples expressed as percent of basal. Each sample was normalized to its 18 S ribosomal RNA content. *, $p < 0.01$ compared with basal levels.

firm the role played by EP4 in mediating COX-2 effects on aromatase activation we silenced the receptor and found a decrease in both aromatase expression and CREB phosphorylation (Fig. 4*F*).

PKA Inhibitor H89 Decreases Aromatase Expression and Activity as a Consequence of Reduced pCREB Activation—Because EP4 receptor activates the PKA transduction pathway, we wanted to test the effect of a specific PKA inhibitor, H89, on aromatase expression. H89 decreased both phosphorylated CREB and aromatase levels in a dose-dependent manner (Fig. 5*A*), concomitantly with enzymatic activity (Fig. 5*B*) and aromatase mRNA (Fig. 5*C*, *left panel*), due to a lower recruitment of pCREB to the PII promoter (Fig. 5*C*, *right panel*), and to increased DAX-1 expression that progressively blocks SF-1 action (data not shown).

PKA involvement can be further demonstrated by the direct measurement of kinase A activity after treatment with the three inhibitors (Fig. 5*D*). As expected H89 caused an 80% inhibition of PKA activity after 4-h treatment. Similarly, NS398 and AH23848 resulted in 40 and 63% inhibition, respectively.

Short Term Inhibition of COX-2/PGE2/PKA Pathway Affects Aromatase Activity at the Post-transcriptional Level—The observation that NS398 almost completely decreases aromatase activity (Fig. 3*B*), with fewer effects on aromatase mRNA levels (Fig. 3*C*), suggests that part of the effects of NS398 on aromatase may be a post-translational modification. To investigate whether COX-2 could regulate aromatase at post-translational levels, we treated R2C cells with NS398, AH23848, and H89 for 1 h and measured aromatase activity (Fig. 6). NS398 resulted in a 53% inhibition, whereas AH23848 and H89 inhibited R2C cells by 25 and 33%, respectively.

Inhibition of the COX-2/PGE2/PKA Pathway Decreases E2 Production and Cyclin E Expression in R2C Cells—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 E2 production (Fig. 7*A*). As previously demonstrated, E2 treatment activates target genes involved in cell cycle regulation such as cyclin E. For this reason we investigated the effects of PGE2 pathway inhibitors on cyclin E expression. NS398 (Fig. 7*B*), AH23848 (Fig. 7*C*), and H89 (Fig. 7*D*) resulted in a decrease in cyclin E expression as a consequence of reduced E2 production.

Inhibition of the COX-2/PGE2/PKA Pathway Decreases R2C Cell Proliferation—As expected, the decreased E2 production resulted in a significant reduction in cell proliferation. Increasing doses of NS398 (Fig. 8*A*), AH23848 (Fig. 8*B*), or H89 (Fig. 8*C*) were associated with a dose-dependent reduction of cell growth. An additional demonstration of the involvement of the COX-2-dependent pathway in controlling E2 production in R2C cells is provided by the evaluation of cell proliferation after knocking down COX-2 in these cells with a specific siRNA (Fig. 8*D*). Thymidine incorporation was reduced by a similar percentage with all amounts of transfected COX-2 siRNA. These findings led us to propose that in tumor Leydig cells the PGE2 derived from COX-2 overexpression could act through an autocrine mechanism in activating aromatase expression, E2 production, and cell proliferation.

DISCUSSION

In the present study, we have demonstrated that COX-2 overexpression in Leydig tumor cells activates the PGE2/PKA transduction pathway, which stimulates consecutive events such as CREB phosphorylation, aromatase expression, and E2-dependent proliferation. In this regard, we have first demon-

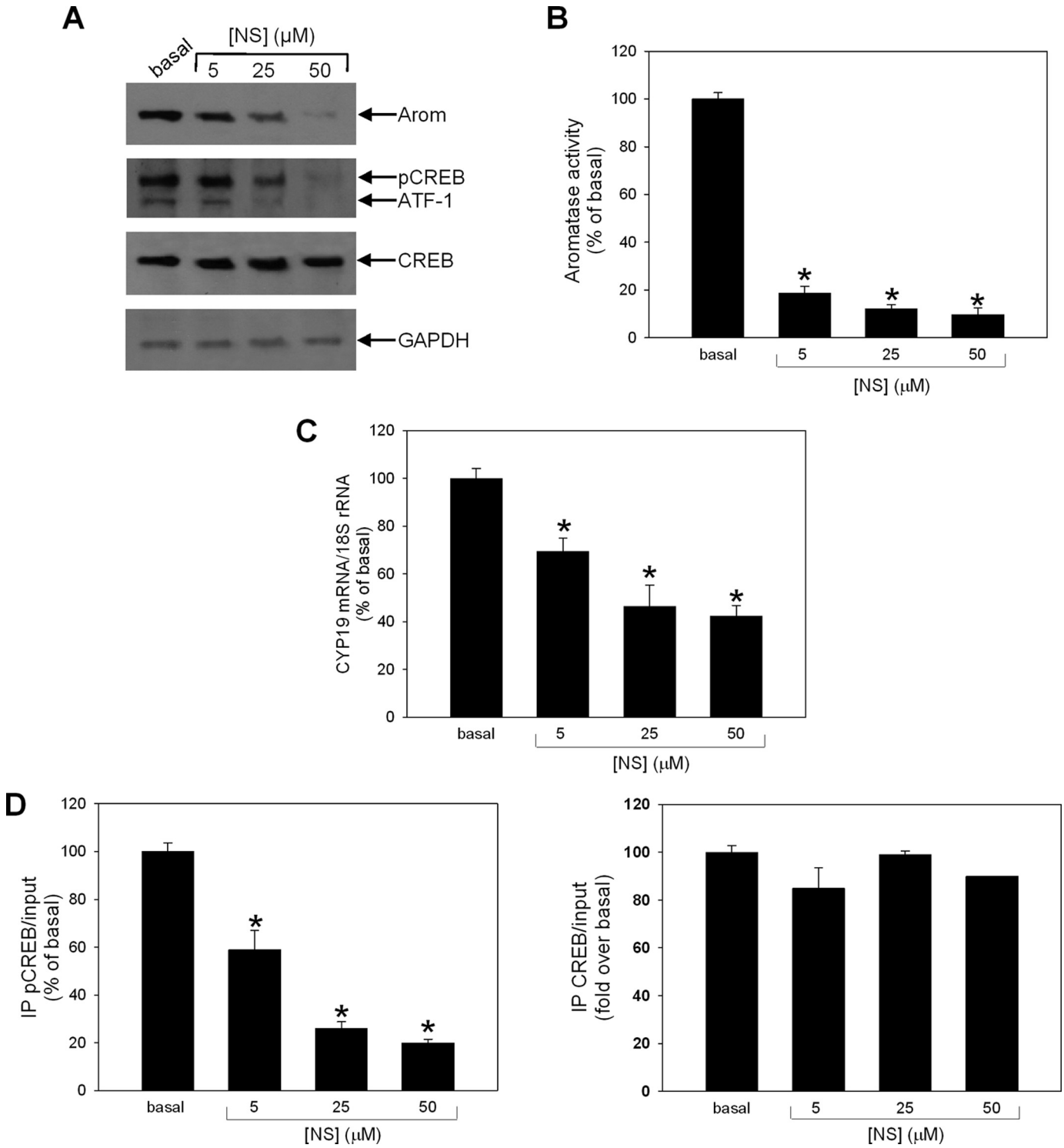

FIGURE 3. **Effects of NS98 on CREB phosphorylation and aromatase expression in R2C cells.** R2C cells were untreated (basal) or treated for 24 h with the indicated doses of NS398. *A*, Western blot analysis of pCREB, CREB, and aromatase were performed on 50 μg of total proteins extracted from R2C cells. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control. *B*, aromatase activity was assessed using the modified tritiated water method. Results obtained were calculated as picomoles of $[^3H]H_2O$ released per hour normalized to the protein content per well (picomoles/h/mg of protein) and expressed as percent of basal. *Columns*, mean of three independent experiments each performed with triplicate samples expressed as percent of basal; *bars*, ±S.E. *C*, total RNA was extracted and real-time RT-PCR was used to analyze mRNA levels of *CYP19*. Data represent the mean ± S.E. of values from three separate RNA samples expressed as percent of basal. Each sample was normalized to its 18 S ribosomal RNA content. *D*, effects of COX-2 inhibitor on pCREB (*left*) or CREB (*right*) binding to aromatase PII promoter in R2C cells. ChIP assays were performed on R2C cells untreated (basal) or treated as indicated. Immunoprecipitated (*IP pCREB*) and total (10% input) DNA were subject to real-time PCR using specific primers. C_t values from immunoprecipitated sample were normalized to the input C_t values. *Columns*, mean of three independent experiments expressed as percent of basal; *bars*, ±S.E. *, $p <$ 0.01 compared with basal.

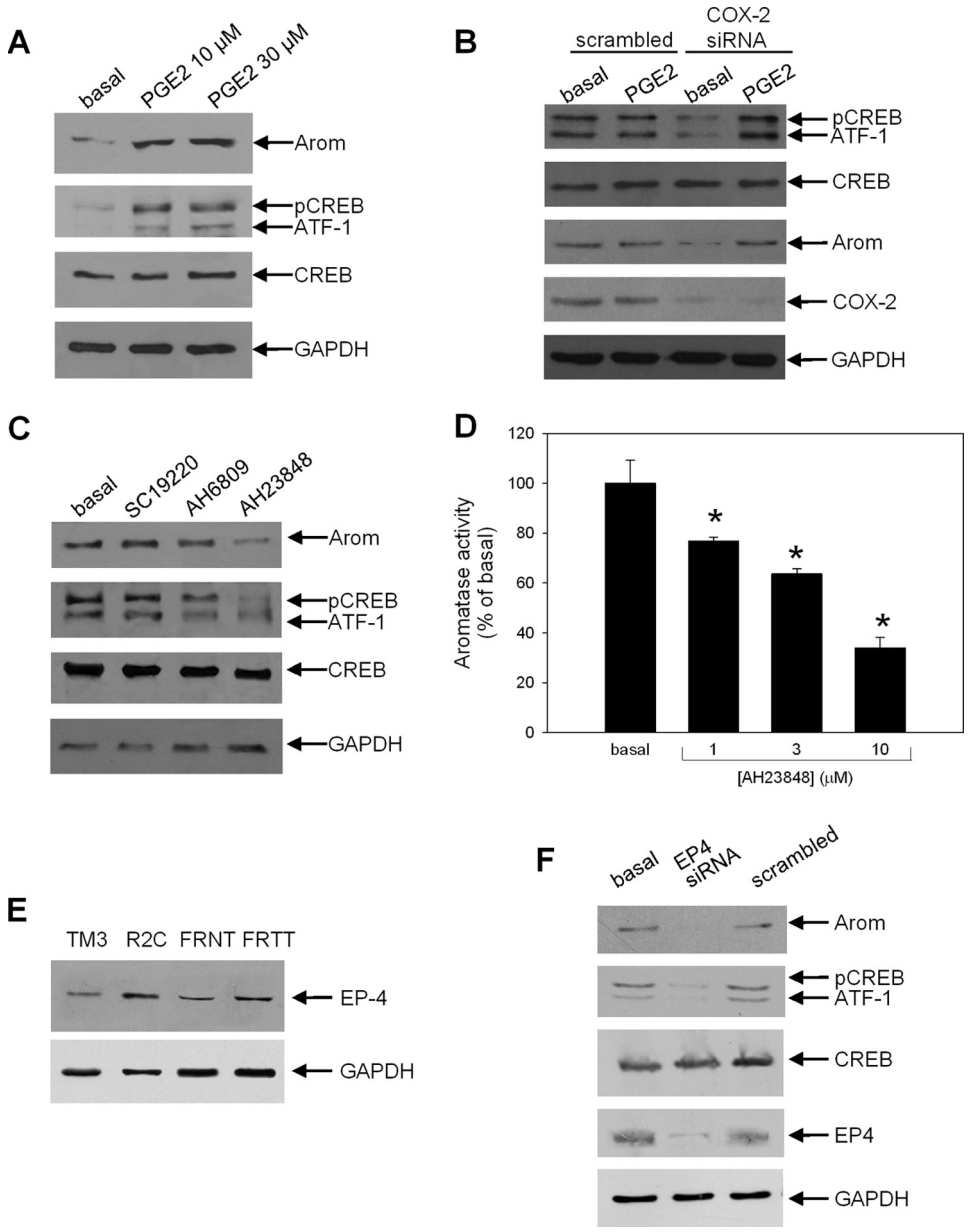

FIGURE 4. **PGE2 activated pathway regulates aromatase expression.** *A*, R2C cells maintained in serum-free medium for 24 h were then left untreated (basal) or treated for 24 h with the indicated doses of PGE2. Western blot analysis of aromatase (*Arom*), pCREB, and CREB were performed on 50 μg of total proteins extracted from R2C cells. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control. *B*, R2C cells were grown for 2 days in the presence of siRNA for COX-2 (100 nM) or scrambled siRNA (100 nM). 24 h after transfection cells were treated with 0.1 μM PGE2 and then lysed for protein extraction. Western blot analysis of aromatase (*Arom*), pCREB, and CREB were performed on 50 μg of total proteins extracted from R2C cells. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control. *C*, R2C cells were untreated (basal) or treated for 24 h with 10 μM of EP inhibitors: SC19220, AH6809, AH23848. Western blot analysis of aromatase (*Arom*), pCREB, and CREB were performed on 50 μg of total proteins extracted from R2C cells. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control. *D*, R2C cells were untreated (basal) or treated for 48 h with increasing doses of AH23848 (1, 3, and 10 μM). Aromatase activity was assessed using the modified tritiated water method. Results obtained were calculated as picomoles of $[^3H]H_2O$ released per hour normalized to the well protein content (picomoles/h/mg of protein) and expressed as percent of basal. *Columns*, mean of three independent experiments each performed with triplicate samples expressed as percent of basal; *bars*, $\pm$S.E. *, $p < 0.01$ compared with basal. *E*, Western blot analysis of EP4 was performed using 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. *F*, R2C cells were grown for 2 days in the presence of siRNA for EP4 (100 nM) or scrambled siRNA (100 nM). Western blot analysis for aromatase (*Arom*), pCREB, CREB, and EP4 was performed on 50 μg of total proteins extracted from R2C cells following siRNA gene silencing. GAPDH was used as a loading control. Results are representative of three independent experiments.

strated, by Western analysis, that both R2C cells and tumor testes from Fischer rats express higher COX-2 levels than normal cell lines and tissues. Thereafter, we have shown, by immunohistochemistry, that COX-2 is highly expressed in human Leydigioma, but is not present in normal testes. Interestingly, COX-2 silencing in R2C cells decreased aromatase expression and CREB phosphorylation, which is involved in the regulation of the aromatase PII promoter in Leydig cells. Moreover, the inhibitory effects observed by knocking down COX-2 were also obtained using a selective COX-2 antagonist, which reduced aromatase activity. These findings together with our previous report (5), showing an increased expression of aromatase in human Leydigioma, provide support for a correlation between COX-2 expression and increased aromatase activity in Leydig cell tumors.

Recent studies have suggested that COX-2 exerts an important role in breast cancer progression. In this respect, it should be noted that COX-2 protein was found to be overexpressed in ~40% of breast tumors (35). Additionally, high COX-2 levels were sufficient to induce mammary tumorigenesis in transgenic mice (36), while the inhibition of COX-2 activity exerted protective effects following the tumorigenesis process in animal models of breast cancer (37). A recent report demonstrated a strong positive correlation between COX-2 and aromatase mRNA expression in human breast cancer tissues (38). In postmenopausal subjects with breast cancer, aromatase activity within the tumor and/or surrounding adipose tissue allows local estrogen production through conversion of androgen precursors (39). Consequently, E2 concentrations within tumor breast tissue can be more than 10 times higher with respect to those measured at circulating levels (40). It is worth noting that elevated aromatase expression within the malignant tissue and/or surrounding

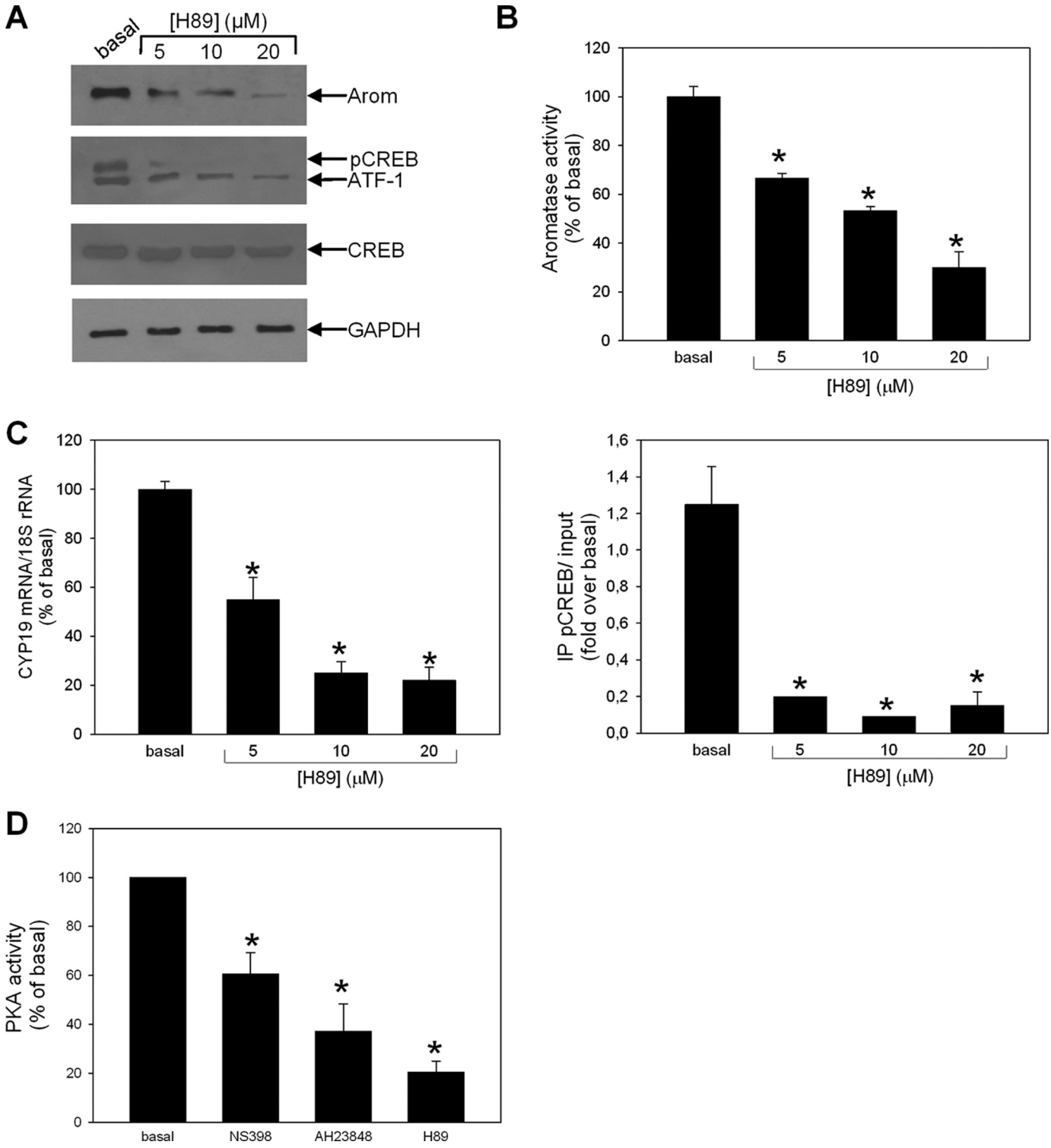

FIGURE 5. **PKA is involved in COX-2/PGE2/pCREB-dependent aromatase expression in R2C cells.** R2C cells were untreated (basal) or treated for 24 h with the indicated doses of H89. *A*, Western blot analysis of aromatase (*Arom*), pCREB, and CREB were performed on 50 μg of total proteins extracted from R2C cells. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control. *B*, aromatase activity was assessed by using the modified tritiated water method. Results obtained were calculated as picomoles of $[^3H]H_2O$ released per hour normalized to the protein content per well (picomoles/h/mg of protein) and expressed as percent of basal. *Columns*, mean of three independent experiments each performed with triplicate samples expressed as percent of basal; *bars*, ±S.E. In *C*: *left panel*, total RNA was extracted, and real-time RT-PCR was used to analyze mRNA levels of *CYP19*. Data represent the mean ± S.E. of values from three separate RNA samples expressed as percent of basal. Each sample was normalized to its 18 S ribosomal RNA content. *Right panel*, effects of COX-2 inhibitor on pCREB binding to aromatase PII promoter in R2C cells. ChIP assays were performed on R2C cells untreated (*bs*) or treated as indicated. Immunoprecipitated (*IP pCREB*) and total (10% input) DNA were subject to real-time PCR using specific primers. C_t values from immunoprecipitated samples were normalized to the input C_t values. Data represent the mean ± S.E. of three independent experiments expressed as percent of basal. *D*, R2C cell lysates were analyzed for PKA activity by non-radioactive *in vitro* PKA assay as described under "Experimental Procedures." R2C cells were treated for 4 h with NS398 (50 μM), AH23848 (10 μM), and H89 (20 μM). Absorbance of phosphorylated peptide was quantified spectrophotometrically. *Columns*, mean of three independent experiments samples expressed as percent of basal; *bars*, ±S.E. *, $p < 0.01$ compared with basal.

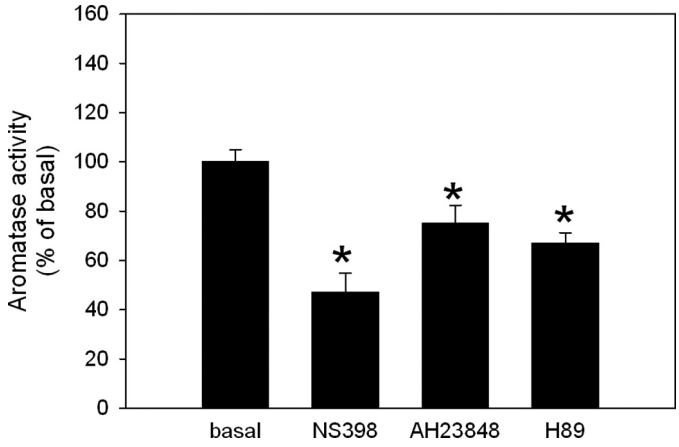

FIGURE 6. **Regulation of aromatase activity by COX-2/PGE2/PKA pathway occurs at the post-transcriptional level.** R2C cells were untreated (basal) or treated for 1 h with NS398 (50 μM), AH23848 (10 μM), or H89 (20 μM). Aromatase activity was assessed using the modified tritiated water method. Results obtained were calculated as picomoles of $[^3H]H_2O$ released per hour normalized to the protein content per well (picomoles/h/mg of protein) and expressed as percent of basal. *Columns*, mean of three independent experiments each performed with duplicate samples expressed as percent of basal; *bars*, ±S.E. *, $p < 0.01$ compared with basal.

stroma may occur in response to tumor-derived stimulatory factors (41) in combination with a switch in *CYP19* gene promoter usage from the specific adipose promoter I.4 to the promoter II active in gonadal tissues (42). In this context, it has been shown that COX-2-derived PGE2 strongly stimulates aromatase promoter II activity (12). These findings suggest that PGE2 may also regulate aromatase activity in other cell types such as Leydig tumor cells where PII drives aromatase expression.

Leydig, Sertoli, and spermatogenic rat testicular cells synthesize PGE2, which has been implicated in the control of steroidogenesis, spermatogenesis, and local immune response (43). Moreover, it has been shown that, in various species, such as the newt, PGE2 increases 17β-estradiol, cAMP, and aromatase activity, while it decreases testosterone, probably due to an increased conversion into estrogens (44). Consistent with findings related to COX-2 and cancer, PGE2 exhibited biological properties facilitating tumor development such as angiogenesis, invasiveness, and inhibition of immune surveillance (45). Importantly R2C cells are able to produce high levels of PGE2 when compared with TM3 normal Leydig cells. In this study we demonstrated the specific role of

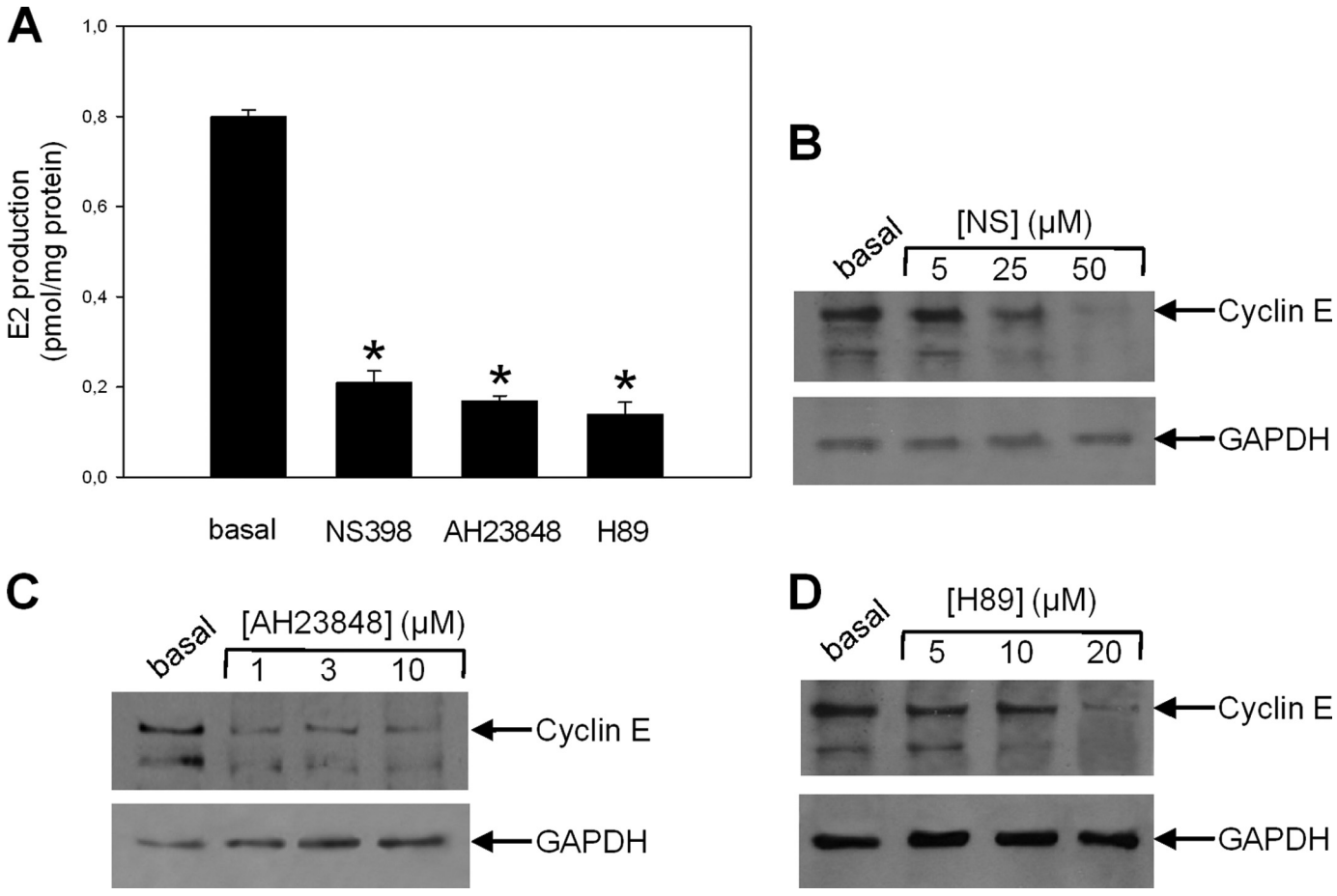

FIGURE 7. **Inhibition of COX-2/PGE2/PKA pathway decreases E2 production and cyclin E expression in R2C cells.** *A*, R2C cells were maintained for 24 h in HAM-F10 medium in the absence (basal) or presence of NS398 (50 μM), AH23848 (10 μM), or H89 (10 μM). E2 content in culture medium was determined by radioimmunoassay and normalized to the cell culture protein content per well (picomoles/mg of protein). Data represent the mean ± S.E. of values from three separate cell culture wells expressed as percent of basal. *, $p < 0.01$ compared with basal. *B–D*, cells were treated in serum-free medium in the absence (basal) or presence of increasing doses of NS398 (5, 25, and 50 μM) (*B*), AH23848 (1, 3, and 10 μM) (*C*), or H89 (5, 10, and 20 μM) (*D*) for 24 h after 24-h starvation. Western blot analysis of cyclin E was performed on 50 μg of total proteins. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control.

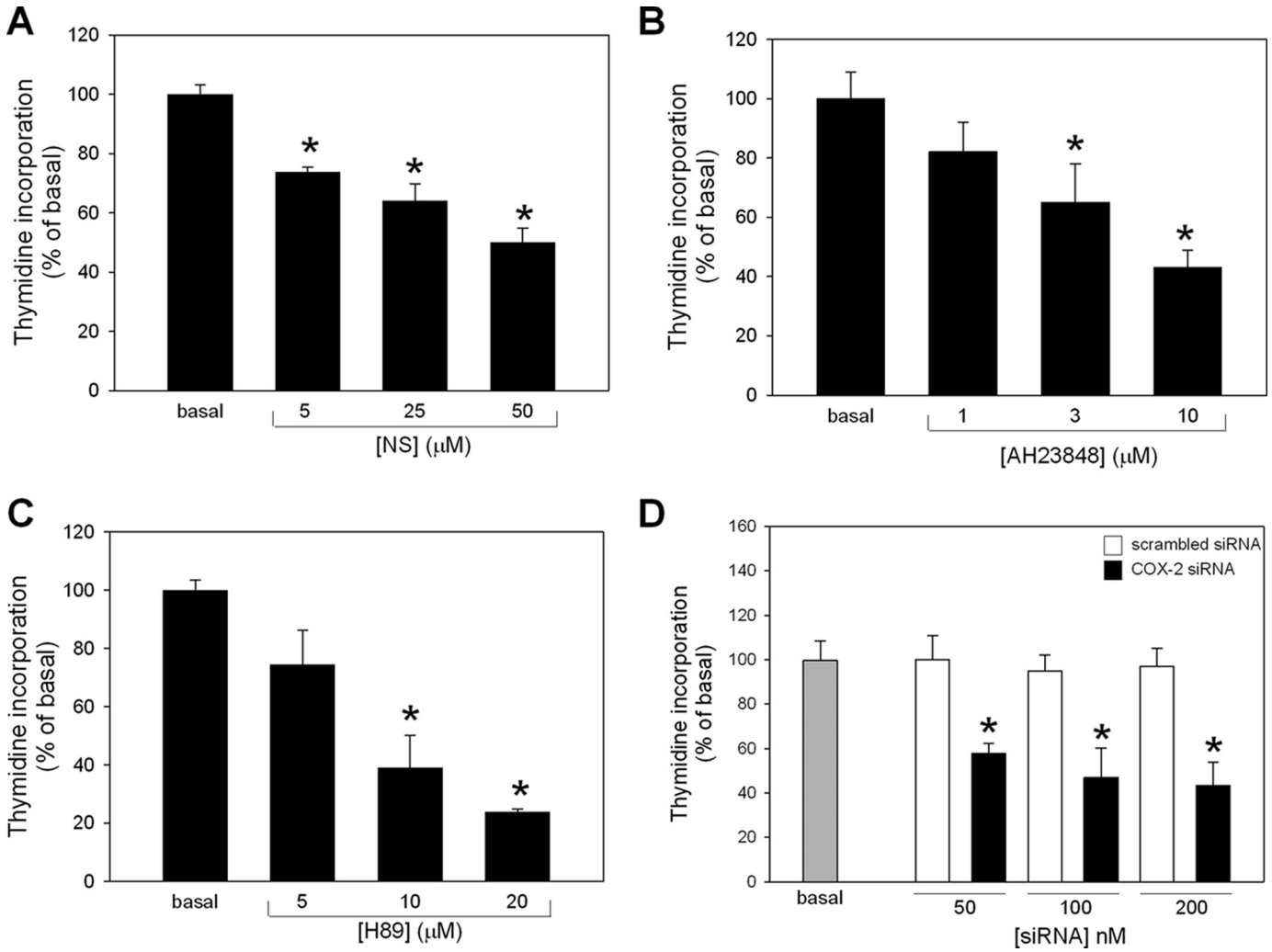

FIGURE 8. **Inhibition of COX-2/PGE2/PKA pathway decreases R2C cell proliferation.** *A–D*, R2C cells were treated for 72 h after 24-h starvation, with the indicated concentrations of NS398, AH23848, or H89. *D*, R2C cells were grown for 2 days in the presence of increasing amounts of COX-2 or scrambled siRNA. R2C cell proliferation was evaluated by [^{3}H]thymidine incorporation analysis. *Columns*, mean of three independent experiments each performed with triplicate samples expressed as percent of basal; *bars*, ±S.E. *, $p < 0.01$ compared with basal.

PGE2 in mediating COX-2 effects on aromatase silencing COX-2 (to block synthesis of any PG) and then treated cells with PGE2, which resulted in a clear induction of aromatase expression.

PGs, produced through COX activity, act by binding to transmembrane G-protein-coupled receptors termed prostanoid receptors, which are expressed as different splice variants (46). Specifically, the expression of the PGE2 receptors EP1, EP2, EP3, and EP4 occurs through modulation of adenylyl cyclase activity, inositol phospholipid hydrolysis, and calcium mobilization (47). EP1 activates PKC while EP2 and EP4 are coupled to PKA. EP3 can either couple negatively to adenylyl cyclase through binding a G_i protein or associate with inositol phospholipid hydrolysis and calcium mobilization. Importantly, in adipose stromal cells the stimulatory effects on aromatase exerted by PGE2 were inhibited using selective antagonists of EP1 and EP2 (48), suggesting that both PKA and protein kinase C transduction pathways are required for aromatase regulation. In the current study, using selective antagonists for the EP subtypes we show that only the EP4 inhibitor AH23848 decreased aromatase expression and activity. We confirmed the involvement of EP4 in regulating aromatase expression using a specific EP4 siRNA. Considering that EP4 signals through PKA, we also blocked this transduction cascade demonstrating a consequent reduction in CREB phosphorylation. In particular, treating R2C cells with the COX-2 inhibitor NS398, the EP4 inhibitor AH23848, and the specific PKA inhibitor H89, we observed that decreased kinase A activity was paralleled by a reduction in CREB phosphorylation and aromatase mRNA and protein levels. Aromatase transcription in Leydig cells is mainly controlled by transcription factors pCREB and SF-1. In a previous study (4) we showed that SF-1 transcriptional regulation is controlled by a transduction pathway that does not involve PKA directly. This indicates that, even in the presence of very low levels of pCREB, as is the case after treating cells with H89, SF-1 can still drive aromatase transcription maintaining the gene expression. This is confirmed by results shown in Fig. 5 (*A* and *C*) demonstrating the presence of aromatase protein and mRNA expression in R2C even after 24 h of treatment with H89.

All these findings imply a regulatory role for the COX-2/PGE2/PKA signaling pathway in aromatase expression. We

also showed that the same pathway could be involved in short term aromatase activation.

It has been postulated that aromatase regulation can occur not only by altering gene expression but also through post-translational modifications (49–51). The observation that NS398, AH23848, and H89 are also able to decrease PKA activity suggests that aromatase activation could occur through PKA-dependent phosphorylation of specific amino acid residues. NS398 showed a more potent effect on aromatase inhibition compared with the other compounds utilized. This could be explained by the observation that NS398, independently of COX-2 inhibition, can also affect the enzymatic activity of kinases involved in post-translational aromatase regulation (52). In a previous study (4), using the same cell system, we showed that PD98089, an inhibitor of MAP kinase, can decrease aromatase activity, without changing mRNA or protein levels. NS398 can also potentially affect MAPK in R2C cells (51, 53), indicating the existence of multiple pathways involved in aromatase activation. This preliminary observation needs to be supported by further studies.

Moreover, it should be taken into account that LH binding to its cognate receptor (LHR) activates a cAMP-PKA transduction cascade that primarily controls Leydig cell function (54). Several observations indicate that constitutively active mutants of LHR could be involved in Leydig cell transformation (55). Indeed, the constitutive activation of the cAMP-PKA pathway in R2C Leydig tumor cells allows for a steroidogenic phenotype (56), which could contribute to aromatase overexpression in Leydig cell tumors. For these reasons we believe that other molecular mechanisms could be involved in constitutive activation of CREB-dependent aromatase activation. However, in the current study all tested inhibitors blocking COX-2, EP4, or PKA reduced the ability of R2C cells to produce E2 resulting in the decreased expression of the estrogen-regulated cyclin E and a reduction in cell proliferation. The same effect was also observed knocking down COX-2 expression, demonstrating that COX-2 overexpression is important for Leydig tumor cell proliferation.

Targeted inhibition of COX-2 and/or PGE2 has been indicated as a potential strategy to stop cancer development. Selective COX-2 inhibitors are used in treatment of colorectal polyps (57) and in women with high grade cervical dysplasia (58). Furthermore, experimental studies have shown that the inhibition of COX-2 or PGE2 leads to *in vivo* tumor reduction in murine lung cancer models (59). Our data in human Leydigioma, demonstrating that COX-2 overexpression is associated with elevated aromatase expression, support the hypothesis that the PGE2/PKA/CREB pathway is also activated in human Leydig cell tumors and suggest the use of COX-2 inhibitors for the treatment of Leydig cell tumors.

In regards to the molecular mechanisms determining COX-2 overexpression in Leydig tumor cells, a recent study has suggested that LH stimulates COX-2 but not COX-1 expression in rat Leydig cells through the cAMP-PKA signaling pathway (60). These findings could explain the COX-2 overexpression observed in Leydig tumor cells. However it has been demonstrated that COX-2 expression can also be regulated by testicular growth factors such as transforming growth factor-α and/or cytokines from macrophages or Sertoli cells (60). Moreover, E2 is also able to induce COX-2 expression in vascular endothelial cells (61). The latter observation, if confirmed in tumor Leydig cells, will open a new interesting perspective regarding a possible autocrine loop triggered by E2 via COX-2/PGE2 determining an increase of aromatase activity and E2 production. Our studies are currently investigating this hypothesis.

In conclusion, the present and our previous study (4) show that Leydig cell tumors are characterized by overexpression of COX-2 and constitutively active insulin-like growth factor I signaling resulting in CREB phosphorylation and SF-1 expression, respectively. Both transcription factors, by binding to promoter II, increase aromatase transcription and, consequently, activity. The excessive local E2 production is able to stimulate the expression of genes involved in cell cycle regulation thereby sustaining Leydig tumor cell proliferation. The observations that both COX-2 and insulin-like growth factor I signaling inhibitors decrease R2C cell proliferation suggest their potential application as new adjuvant therapies in Leydig tumor cell treatment.

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REFERENCES

1. Hawkins, C., and Miaskowski, C. (1996) *Oncol. Nurs. Forum* **23,** 1203–1211; quiz 1212–1213
2. Simpson, E. R., Mahendroo, M. S., Means, G. D., Kilgore, M. W., Hinshelwood, M. M., Graham-Lorence, S., Amarneh, B., Ito, Y., Fisher, C. R., and Michael, M. D. (1994) *Endocr. Rev.* **15,** 342–355
3. Fowler, K. A., Gill, K., Kirma, N., Dillehay, D. L., and Tekmal, R. R. (2000) *Am. J. Pathol.* **156,** 347–353
4. Sirianni, R., Chimento, A., Malivindi, R., Mazzitelli, I., Andò, S., and Pezzi, V. (2007) *Cancer Res.* **67,** 8368–8377
5. Carpino, A., Rago, V., Pezzi, V., Carani, C., and Andò, S. (2007) *Eur. J. Endocrinol.* **157,** 239–244
6. O'Donnell, L., Robertson, K. M., Jones, M. E., and Simpson, E. R. (2001) *Endocr. Rev.* **22,** 289–318
7. Young, M., Lephart, E. D., and McPhaul, M. J. (1997) *J. Steroid Biochem. Mol. Biol.* **63,** 37–44
8. Pezzi, V., Sirianni, R., Chimento, A., Maggiolini, M., Bourguiba, S., Delalande, C., Carreau, S., Andò, S., Simpson, E. R., and Clyne, C. D. (2004) *Endocrinology* **145,** 2186–2196
9. Young, M., and McPhaul, M. J. (1998) *Endocrinology* **139,** 5082–5093
10. Carlone, D. L., and Richards, J. S. (1997) *Mol. Endocrinol.* **11,** 292–304
11. Song, K. H., Park, Y. Y., Park, K. C., Hong, C. Y., Park, J. H., Shong, M., Lee, K., and Choi, H. S. (2004) *Mol. Endocrinol.* **18,** 1929–1940
12. Zhao, Y., Agarwal, V. R., Mendelson, C. R., and Simpson, E. R. (1996) *Endocrinology* **137,** 5739–5742
13. Smith, W. L., and Marnett, L. J. (1991) *Biochim. Biophys. Acta* **1083,** 1–17
14. Chandrasekharan, N. V., and Simmons, D. L. (2004) *Genome Biol.* **5,** 241–247
15. Tsuji, S., Tsujii, M., Kawano, S., and Hori, M. (2001) *J. Exp. Clin. Cancer Res.* **20,** 117–129
16. Sano, H., Kawahito, Y., Wilder, R. L., Hashiramoto, A., Mukai, S., Asai, K., Kimura, S., Kato, H., Kondo, M., and Hla, T. (1995) *Cancer Res.* **55,** 3785–3789
17. Ristimäki, A., Honkanen, N., Jänkälä, H., Sipponen, P., and Härkönen, M. (1997) *Cancer Res.* **57,** 1276–1280
18. 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., and Sata, M. (1999) *Hepatology* **29,** 688–696
19. Hida, T., Yatabe, Y., Achiwa, H., Muramatsu, H., Kozaki, K., Nakamura, S., Ogawa, M., Mitsudomi, T., Sugiura, T., and Takahashi, T. (1998) *Cancer Res.* **58,** 3761–3764
20. Zimmermann, K. C., Sarbia, M., Weber, A. A., Borchard, F., Gabbert, H. E., and Schrör, K. (1999) *Cancer Res.* **59,** 198–204
21. Tucker, O. N., Dannenberg, A. J., Yang, E. K., Zhang, F., Teng, L., Daly, J. M., Soslow, R. A., Masferrer, J. L., Woerner, B. M., Koki, A. T., and Fahey, T. J., 3rd (1999) *Cancer Res.* **59,** 987–990
22. Hwang, D., Scollard, D., Byrne, J., and Levine, E. (1998) *J. Natl. Cancer Inst.* **90,** 455–460
23. Brueggemeier, R. W., Quinn, A. L., Parrett, M. L., Joarder, F. S., Harris, R. E., and Robertson, F. M. (1999) *Cancer Lett* **140,** 27–35
24. Díaz-Cruz, E. S., Shapiro, C. L., and Brueggemeier, R. W. (2005) *J. Clin. Endocrinol. Metab.* **90,** 2563–2570
25. Prosperi, J. R., and Robertson, F. M. (2006) *Prostaglandins Other Lipid Mediat.* **81,** 55–70
26. Frungieri, M. B., Weidinger, S., Meineke, V., Köhn, F. M., and Mayerhofer, A. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99,** 15072–15077
27. Hase, T., Yoshimura, R., Matsuyama, M., Kawahito, Y., Wada, S., Tsuchida, K., Sano, H., and Nakatani, T. (2003) *Eur. J. Cancer* **39,** 2043–2049
28. Coleman, G. L., Barthold, W., Osbaldiston, G. W., Foster, S. J., and Jonas, A. M. (1977) *J. Gerontol.* **32,** 258–278
29. Davis, T. L., and Sharif, N. A. (2000) *Br. J. Pharmacol.* **130,** 1919–1926
30. Woodward, D. F., Pepperl, D. J., Burkey, T. H., and Regan, J. W. (1995) *Biochem. Pharmacol.* **50,** 1731–1733
31. Fulton, A. M., Zhang, S. Z., and Chong, Y. C. (1991) *Cancer Res.* **51,** 2047–2050
32. Inoue, H., Tsujisawa, T., Fukuizumi, T., Kawagishi, S., and Uchiyama, C. (1999) *J. Endocrinol.* **161,** 231–236
33. Chandras, C., Harris, T. E., Bernal, A. L., Abayasekara, D. R., and Michael, A. E. (2007) *J. Endocrinol.* **194,** 595–602
34. Lephart, E. D., and Simpson, E. R. (1991) *Methods Enzymol.* **206,** 477–483
35. Ristimäki, A., Sivula, A., Lundin, J., Lundin, M., Salminen, T., Haglund, C., Joensuu, H., and Isola, J. (2002) *Cancer Res.* **62,** 632–635
36. Liu, C. H., Chang, S. H., Narko, K., Trifan, O. C., Wu, M. T., Smith, E., Haudenschild, C., Lane, T. F., and Hla, T. (2001) *J. Biol. Chem.* **276,** 18563–18569
37. Alshafie, G. A., Abou-Issa, H. M., Seibert, K., and Harris, R. E. (2000) *Oncol. Rep.* **7,** 1377–1381
38. Salhab, M., Singh-Ranger, G., Mokbel, R., Jouhra, F., Jiang, W. G., and Mokbel, K. (2007) *J. Surg. Oncol.* **96,** 424–428
39. Simpson, E. R., Clyne, C., Rubin, G., Boon, W. C., Robertson, K., Britt, K., Speed, C., and Jones, M. (2002) *Annu. Rev. Physiol.* **64,** 93–127
40. van Landeghem, A. A., Poortman, J., Nabuurs, M., and Thijssen, J. H. (1985) *Cancer Res.* **45,** 2900–2906
41. Harada, N. (1997) *J. Steroid Biochem. Mol. Biol.* **61,** 175–184
42. Agarwal, V. R., Bulun, S. E., Leitch, M., Rohrich, R., and Simpson, E. R. (1996) *J. Clin. Endocrinol. Metab.* **81,** 3843–3849
43. Winnall, W. R., Ali, U., O'Bryan, M. K., Hirst, J. J., Whiley, P. A., Muir, J. A., and Hedger, M. P. (2007) *Biol. Reprod.* **76,** 759–768
44. Gobbetti, A., and Zerani, M. (2002) *J. Exp. Zool.* **293,** 73–80
45. Sheng, H., Shao, J., Washington, M. K., and DuBois, R. N. (2001) *J. Biol. Chem.* **276,** 18075–18081
46. Coleman, R. A., Eglen, R. M., Jones, R. L., Narumiya, S., Shimizu, T., Smith, W. L., Dahlén, S. E., Drazen, J. M., Gardiner, P. J., Jackson, W. T., *et al.* (1995) *Adv. Prostaglandin Thromboxane Leukot. Res.* **23,** 283–285
47. Breyer, R. M., Bagdassarian, C. K., Myers, S. A., and Breyer, M. D. (2001) *Annu. Rev. Pharmacol. Toxicol.* **41,** 661–690
48. Richards, J. A., and Brueggemeier, R. W. (2003) *J. Clin. Endocrinol. Metab.* **88,** 2010–2816
49. Balthazart, J., Baillien, M., Charlier, T. D., and Ball, G. F. (2003) *Eur. J. Neurosci.* **17,** 1591–1606
50. Balthazart, J., Baillien, M., Charlier, T. D., Cornil, C. A., and Ball, G. F. (2003) *J. Steroid Biochem. Mol Biol.* **86,** 367–379
51. Yue, W., Wang, J. P., Conaway, M. R., Li, Y., and Santen, R. J. (2003) *J. Steroid Biochem. Mol. Biol.* **86,** 265–274
52. Su, B., Díaz-Cruz, E. S., Landini, S., and Brueggemeier, R. W. (2008) *Steroids* **73,** 104–111
53. Pan, M. R., Chang, H. C., and Hung, W. C. (2008) *Cell Signal.* **20,** 1134–1141
54. Dufau, M. L., Winters, C. A., Hattori, M., Aquilano D., Barañao, J. L., Nozu, K., Baukal, A., and Catt, K. J. (1984) *J. Steroid Biochem.* **20,** 161–173
55. Ascoli, M. (2007) *Mol. Cell Endocrinol.* **260–262,** 244–248
56. Jo, Y., King, S. R., Khan, S. A., and Stocco, D. M. (2005) *Biol. Reprod.* **73,** 244–255
57. Bertagnolli, M. M., Eagle, C. J., Zauber, A. G., Redston, M., Solomon, S. D., Kim, K., Tang, J., Rosenstein, R. B., Corle, D., Hess, T. M., Woloj, G. M., Boisserie, F., Anderson, W. F., Viner, J. L., Bagheri, D., Burn, J., Chung, D. C., Dewar, T., Foley, T. R., Hoffman, N., Macrae, F., Pruitt, R. E., Saltzman, J. R., Salzberg, B., Sylwestrowicz, T., Gordon, G. B., and Hawk, E. T. (2006) *N. Engl. J. Med.* **355,** 873–884
58. Farley, J. H., Truong, V., Goo, E., Uyehara, C., Belnap, C., and Larsen, W. I. (2006) *Gynecol. Oncol.* **103,** 425–430
59. Stolina, M., Sharma, S., Lin, Y., Dohadwala, M., Gardner, B., Luo, J., Zhu, L., Kronenberg, M., Miller, P. W., Portanova, J., Lee, J. C., and Dubinett, S. M. (2000) *J. Immunol.* **164,** 361–370
60. Chen, H., Luo, L., Liu, J., and Zirkin, B. R. (2007) *Endocrinology* **148,** 735–742
61. Pedram, A., Razandi, M., Aitkenhead, M., Hughes, C. C., and Levin, E. R. (2002) *J. Biol. Chem.* **277,** 50768–50775

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ORIGINAL ARTICLE

Gper and ESRs are expressed in rat round spermatids and mediate oestrogen-dependent rapid pathways modulating expression of cyclin B1 and Bax

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Summary

Spermatogenesis is a precisely controlled and timed process, comprising mitotic divisions of spermatogonia, meiotic divisions of spermatocytes, maturation and differentiation of haploid spermatids giving rise to spermatozoa. It is well known that the maintenance of spermatogenesis is controlled by gonadotrophins and testosterone, the effects of which are modulated by a complex network of locally produced factors, including oestrogens. However, it remains uncertain whether oestrogens are able to activate rapid signalling pathways directly in male germ cells. Classically, oestrogens act by binding to oestrogen receptors (ESRs) 1 and 2. Recently, it has been demonstrated that rapid oestrogen action can also be mediated by the G-protein-coupled oestrogen receptor 1 (Gper). The aim of the present study was to investigate ESRs and Gper expression in primary cultures of adult rat round spermatids (RS) and define if oestradiol (E2) is able to activate, through these receptors, pathways involved in the regulation of genes controlling rat RS apoptosis and/or maturation. In this study, we demonstrated that rat RS express ESR1, ESR2 and Gper. Short-time treatment of RS with E2, the selective Gper agonist G1 and the selective ESR1 and ERβ agonists, 4,4′,4″-(4-propyl-[1H]pyrazole-1,3,5-triyl) trisphenol (PPT) and 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN), respectively, determined activation of Extra-cellular signal-regulated kinase (ERK1/2) through the involvement of epidermal growth factor receptor transactivation. In addition, we investigated the effects of ESRs and Gper pathway activation on factors involved in RS maturation. Expression of cyclin B1 mRNA was downregulated by E2, G1 and PPT, but not by DPN. A concomitant and inverse regulation of the pro-apoptotic factor Bax mRNA expression was observed in the same conditions, with DPN being the only one determining an increase in this factor expression. Collectively, these data demonstrate that E2 activates, through ESRs and Gper, pathways involved in the regulation of genes controlling rat RS apoptosis and differentiation such as cyclin B1 and Bax.

Introduction

The maintenance of spermatogenesis is controlled by gonadotrophins and testosterone, the effects of which are modulated by a complex network of endocrine and paracrine factors, among them oestrogens. Biological effects of oestradiol (E2) result from the activation and subsequent interaction of oestrogen receptor (ESR)1 and ESR2 with the genome. ESRs are widely distributed in the male reproductive tract (Fisher *et al.*, 1997; Hess & Carnes, 2004; Yamashita, 2004; Sierens *et al.*, 2005); ESR1 immunostaining has been detected in Leydig and peritubular myoid cells, but not in Sertoli or germ cells (Fisher *et al.*, 1997; Hess & Carnes, 2004; Sierens *et al.*,

2005; Carreau *et al.*, 2006; Carreau & Hess, 2010). However, other studies have reported different results (Pelletier *et al.*, 2000; Mutembei *et al.*, 2005; Ramesh *et al.*, 2007). ESR2 expression has been detected in multiple cell types, including Sertoli cells, and in some germ cells (Saunders *et al.*, 1998; Zhou *et al.*, 2002). Whereas it is generally agreed that both ER isoforms are expressed by the epithelial cells of the efferent ductules and epididymis, there are conflicting reports in the literature regarding their localization in the testis, possibly because of the specificity of the antisera used in immunocytochemistry experiments (O'Donnell *et al.*, 2001). In addition to the classic genomic mechanism mediated by ESR1 and ESR2, oestrogens exert rapid non-genomic action initiated at cell surface that can occur within minutes (Levin & Pietras, 2008).

Oestrogen rapid signalling involves a series of cell type-dependent events that include mobilization of second messengers, interaction with membrane receptors such as insulin-like growth factor 1 receptor (IGF1R) and epidermal growth factor receptor (EGFR), and stimulation of effector molecules, such as the Src family of tyrosine kinases and the phosphatidylinositol-3-kinase, the serine/threonine protein kinase (AKT) and the mitogen-activated protein kinases (MAPKs; Bjornstrom & Sjoberg, 2005; Hewitt *et al.*, 2005a; Song *et al.*, 2005; Song & Santen, 2006; Manavathi & Kumar, 2006).

Several studies suggest that rapid responses to oestrogens can be mediated by classical receptors localized to the plasma membrane (Levin, 2005; Harrington *et al.*, 2006; Pedram *et al.*, 2006), or by transmembrane proteins other than ESRs. Recently, our study and other studies identified a transmembrane oestrogen-binding protein, the G-protein-coupled oestrogen receptor 1 (Gper), which is able to mediate oestrogen action (Filardo *et al.*, 2002; Maggiolini *et al.*, 2004; Revankar *et al.*, 2005; Prossnitz & Maggiolini, 2009).

Gper has been identified in a variety of human and rodent oestrogen target tissues (Brailoiu *et al.*, 2007; Chagin & Savendahl, 2007; Sakamoto *et al.*, 2007; Wang *et al.*, 2007), including the testis (Isensee *et al.*, 2009). Immunocytochemical studies have identified the intracellular localization of Gper in the endoplasmic reticulum, Golgi apparatus (Revankar *et al.*, 2005) and plasma membrane (Funakoshi *et al.*, 2006). It has been shown that Gper in response to E2 induces the release of surface-bound membrane-anchored heparin-binding EGF-like growth factor (proHB-EGF; Filardo *et al.*, 2000), determining the EGFR/MAPK signalling axis activation. Our recent studies demonstrated that Gper is expressed in mouse testes, in a GC-1 spermatogonia mouse cell line (Sirianni *et al.*, 2008), in rat testes and in rat pachytene spermatocytes (Chimento *et al.*, 2010).

Some studies in vivo and in vitro revealed that oestrogens can act as germ cell survival factor and that this effect is dose-dependent (Pentikäinen *et al.*, 2000). For example, E2 prevents apoptosis of germ cells within human seminiferous tubules in vitro in the absence of gonadotropins (Pentikäinen *et al.*, 2000). Moreover, a beneficial effect for oestrogens on stages 1–6 spermatids (differentiation/elongation) has been reported (D'Souza *et al.*, 2005). However, pro-apoptotic effects of E2 on spermatogenesis have also been observed. Mishra & Shaha (2005) demonstrated that E2-induced apoptosis in different germ cells occurred in vitro in the absence of somatic cells, evidencing independent (not mediated by Sertoli cells) capability of germ cells to respond to oestrogens activating apoptosis. Other studies performed in vivo demonstrated that E2 administration determines germ cell apoptosis in immature and adult rats (Blanco-Rodriguez & Martinez-Garcia, 1997; Walczak-Jedrzejowska *et al.*, 2007). These results and others coming from studies performed on human, rat, mouse and bank vole germ cells (Ebling *et al.*, 2000; Pentikäinen *et al.*, 2000; Gancarczyk *et al.*, 2004; D'Souza *et al.*, 2005) show that oestrogens can determine different effects on different species and/or germ cell types. This phenomenon could be explained by different patterns of expression and/or action of oestrogen receptors among different species and different germ cell types.

Spermatogenesis requires a complex regulation of cell proliferation and cell death, and apoptosis appears to be the underlying major mechanism of germ cell death during normal spermatogenesis in various mammals including rats, hamsters, mice and humans (Sinha Hikim & Swerdloff, 1999). Cell proliferation is controlled by genes involved in the regulation of the cell cycle. In germ cells, the principal regulatory proteins are cyclins (Yu & Wu, 2008), which are categorized according to their appearance during the cell cycle. B-type cyclins function at the G2/M transition of the cell cycle. In the rat testis, cyclin B and its kinase are associated with spermatogonial/early spermatocyte proliferation (Godet *et al.*, 2000). However, high levels of cyclin B1 transcripts in the rat post-meiotic male germ cells were found. In fact, using in situ hybridization, it has been shown that cyclin B1 stage-specific expression during spermatogenesis was highest in late pachytene spermatocytes and early round spermatids (RS; Gromoll *et al.*, 1997). These results may implicate a function for cyclin B1, which does not involve cell cycle progression; rather, cyclin B1 could be involved in spermatid differentiation and apoptosis regulation, as it is a key regulator of apoptosis in response to DNA damage (Chapman & Wolgemuth, 1993; Gromoll *et al.*, 1997; Porter *et al.*, 2003; Kaushal & Bansal, 2007).

Germ cell apoptosis was detected in all testes and was mainly seen in primary spermatocytes and spermatids (Oldereid *et al.*, 2001). The members of the Bcl-2 family of proteins have been reported to be involved in the regu-

lation of apoptosis in various cell types, and may either inhibit (Bcl-2, Bcl-x_L, Mcl-1, Bcl-w) or promote apoptosis (Bax, Bcl-x_S, Bak, Bad; Sinha Hikim & Swerdloff, 1999). In the testis, upregulation of Bax is a feature of germ cell apoptosis in vitro (Sofikitis *et al.*, 2008). Moreover, Bax shows a preferential expression in human RS and could be involved in the regulation of spermatid apoptosis and differentiation into sperm cells (Oldereid *et al.*, 2001).

The aim of this study was to investigate the expression of ESRs and Gper in primary cultures of rat RS and the ability of E2 to activate, through these receptors, pathways involved in the regulation of genes controlling rat RS apoptosis and differentiation such as cyclin B1 and Bax.

Materials and methods

Animals

Sprague–Dawley rats were purchased from the breeding company CERJ – le Genest Saint-Isle, France). They were bred under standard conditions (12 h light : 12 h dark cycle and controlled room temperature) with standard rat food and water ad libitum. All animal procedures were carried out in accordance with French Government Regulations (Services Vétérinaires de la Santé et de la Production Animale, Ministère de l'Agriculture) and approved by the local ethical committee of the university.

Purification of RS

Testicular mixed germ cells of 90-day-old rats were obtained after treatment with trypsin–DNase (Bourguiba *et al.*, 2003); germ cell suspension was washed in phosphate-buffered saline (PBS) supplemented with 6 mM of glucose (Merck, Whitehouse Station, NJ, USA) and 10 mM of pyruvic acid (Sigma-Aldrich Italia S.r.l., Milan, Italy). Germ cells were filtered through fine nylon mesh to hold Sertoli cells and then through glass wool to remove spermatozoa. The separation of the different cell types was realized by unit gravity sedimentation through a bovine serum albumin or BSA (Roche Diagnostics, Indianapolis, IN, USA) gradient (0.2–2.75%) in a Sta-Put apparatus (Bellve *et al.*, 1977). Fractions enriched with RS were identified and washed with PBS buffer as described previously (Levallet *et al.*, 1998). RS (9×10^6/mL) were incubated for 12 h in Ham's F-12/Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, St Louis, MO, USA) with 2% Ultroser, serum substitutes without steroids (Ciphergen, Le Raincy, France), containing $NaHCO_3$ (2.44 g/L), Hepes (3.57 g/L), streptomycin (100 mg/L), penicillin (100 000 U/L) and fungizone (250 μg/L), and supplemented with 10 mM of pyruvic acid (Sigma) and 6 mM of glucose (Merck). To estimate the contamination of germ cell fractions by Leydig cells, a 3β-hydroxysteroid dehydrogenase histochemical staining (Klinefelter *et al.*, 1987), a specific marker of Leydig cells, was realized, showing <1% contamination. Cell preparations were assessed for Sertoli cell contamination as described previously (Levallet *et al.*, 1998). Germ cell preparation purity was higher than 95% (the major contamination was by other germ cells).

Treatments were performed at different times using ICI 182780 (ICI; Tocris Bioscience, Ellisville, MO, USA), PD98059 (Sigma), AG1478 (Sigma), E2 (Sigma), G1 (Merck KGaA, Frankfurt, Germany), 2,3-*bis*(4-hydroxyphenyl)-propionitrile (DPN; Tocris Bioscience) and 4,4′,4″-(4-propyl-[1H]pyrazole-1,3,5-triyl) trisphenol (PPT; Tocris Bioscience) solubilized at the appropriate concentration in Ham's F-12/DMEM without phenol red.

Western blot analysis

After treatments, RS were lysed in ice-cold Ripa buffer containing protease inhibitors [20 mM Tris, 150 mM NaCl, 1% Igepal, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% sodium dodecyl sulphate (SDS), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.15 U/mL aprotinin and 10 μM leupeptin] for protein extraction. Lysates were centrifuged at 16 532 ***g*** for 10 min at 4 °C. Protein content was determined by the Bradford method. The proteins were separated on 11% SDS/polyacrylamide gel and then electroblotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Blots were incubated overnight (O/N) at 4 °C with (i) anti-Gper polyclonal antibody (1 : 1000; MBL International Corporation, Woburn, MA, USA), (ii) anti-ESR1 (F-10) antibody (1 : 4000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), (iii) anti-ESR2 (H-150) antibody (1 : 1000; Santa Cruz Biotechnology), (iv) anti-ERK antibody (1 : 1000; Cell Signaling Technology, Celbio, Milan, Italy), (v) anti-pERK antibody (1 : 1000; Cell Signaling Technology). Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Pharmacia Biotech) and immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Biosciences, Cologno Monzese, Italy). To assure equal loading of proteins, membranes were stripped and incubated overnight with an anti-GAPDH antibody (1 : 3000; Santa Cruz Biotechnology).

RNA extraction, RT-PCR and real-time RT-PCR

The TRizol RNA isolation system [Invitrogen S.r.l., San Giuliano Milanese (MI), Italy] was used to extract RNA from RS untreated or treated for 6 h. Each RNA sample was treated with DNase I (Ambion, Austin, TX, USA), and purity and integrity of the RNA were confirmed spectroscopically and by gel electrophoresis before use. One

microgram of total RNA was reverse transcribed (RT) in a final volume of 30 μL using the ImProm-II Reverse Transcriptase System Kit (Promega Italia S.R.L., Milan, Italy). cDNAs were directly used for polymerase chain reaction (PCR) or diluted 1 : 3 in nuclease-free water to be used for real-time PCR. Samples were aliquoted and stored at −20 °C. PCR amplification was performed using 1.5 U of Taq DNA polymerase (Promega Italia S.r.l.) in PCR buffer containing 200 μM of dNTP, 1.5 mM of $MgCl_2$ and 25 pmol of each primer in a total volume of 50 μL. For ESR1, the PCR conditions were 30 s at 95 °C, 30 s at 60 °C and 45 s at 72 °C for 40 cycles using the following primers: forward, 5′-AATTCTGACAATCGACGCCAG-3′; reverse, 5′-GTGCTTCAACATTCTCCCTCCTC-3′. For ESR1, the PCR conditions were 1 min at 94 °C, 1 min at 56 °C and 2 min at 72 °C for 40 cycles using the following primers: forward, 5′-GAAGCTGAACCACCCAATGT-3′; reverse, 5′-CAGTCCCACCATTAGCACCT-3′. For Gper, the PCR conditions were 30 s at 95 °C, 30 s at 60 °C and 45 s at 72 °C for 35 cycles using the following primers: forward, 5′-TGGGGACCTCTCTGAACATC-3′; reverse, 5′-GCAGGAAGAGGGACATGAAG-3′ amplifying mouse and rat transcripts. L19 ribosomal protein mRNA was used as housekeeping gene; PCR conditions were 30 s at 95 °C, 30 s at 60 ° C and 45 s at 72 °C for 30 cycles using the following primers: forward, 5′-GAAATCGCCAATGCCA ACTC-3′; reverse, 5′-ACCTTCAGGTACAGGCTGTG-3′. PCR products were analysed by electrophoresis on a 2% agarose gel stained with ethidium bromide (Sigma-Aldrich Italia S.r.l.).

Real-time PCR reactions were performed in the iCycler iQ Detection System (Bio-Rad, Hercules, CA, USA), using 0.1 μM of each primer, in a total volume of 30 μL reaction mixture following the manufacturer's recommendations. The nucleotide sequences of the primers for *Ccnb1* (cyclin B1) were: forward, 5′-CCAGAGGTGGAACTG GATG-3′; reverse, 5′-GGGCTTGGAGAGGGAGTATC-3′. For Bax amplification, the following primers were used: forward, 5′-CGTGGTTGCCCTCTTCTACTTT-3′; reverse, 5′-GATCAGCTCGGGCACTTTAGTG-3′.

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 GAPDH content. The nucleotide sequences of the primers for GAPDH were: forward, 5′-TGCACCACCACCTGCT TAGC-3′; reverse, 5′-GGCATGGACTGTGGTCATGAG-3′. The relative gene expression was calculated by the $\Delta\Delta C_t$ method as described previously (Sirianni *et al.*, 2008). The C_t values obtained for the target gene were normalized to the endogenous control (GAPDH) to obtain the ΔC_t values. The $\Delta\Delta C_t$ was calculated using basal (untreated sample) as calibrator, where $\Delta\Delta C_t = \Delta C_t$ (test sample) $- \Delta C_t$ (calibrator).

Immunofluorescence assay (or staining)

RS were first sedimented on microscope slides for 30 min at 4 °C in an humidified atmosphere before being permeabilized with cooled methanol (Sigma-Aldrich Italia S.r.l.) for 10 min until evaporation and then fixed at room temperature using fresh paraformaldehyde (3% in PBS) (Sigma-Aldrich Italia S.r.l.). Before incubation with primary antibody, the slides were washed thrice with PBS (Gibco, Carlsbad, CA, USA) and non-specific binding of immunoglobulin G (IgG) was blocked with 3% BSA in PBS for 20 min. The slides were incubated for 48 h at 4 °C with rabbit anti-Gper antibody (MBL International Corp., Woburn, MA, USA) at 1 : 50 dilution in 3% BSA in PBS or with rabbit anti-ESR1 antibody (MC-20; Santa Cruz). On the third day, Gper slides were washed with PBS and then incubated with goat anti-rabbit Ig (Amersham Pharmacia Biotech) at 1 : 400 dilution in PBS for 45 min at room temperature; then the slides were incubated at room temperature with streptavidine-fluoresceine (FITC; Amersham Pharmacia Biotech) diluted 1 : 100 in PBS for 30 min. For ESR1, the slides were incubated for 45 min with anti-rabbit IgG linked to Alexa F488 (Invitrogen). After being washed with PBS, all slides were cover-slipped with mounting medium containing 4′-6-diamidino-2-phenylindole (DAPI) (Santa Cruz Biotechnology). Control staining was performed on adjacent serial slides and consisted in replacing the primary antibody with 3% BSA in PBS. The cellular expression and localization of Gper and ERS1 were observed using Olympus confocal laser scanning biological microscope (Fluoview FV1000, Olympus America Inc., Center Valley, PA, USA).

Data analysis and statistical methods

Data from triplicate experiments were pooled and analysed using one-way ANOVA with Fisher's least significant differences multiple comparison method, using SIGMASTAT version 3.0 (SPSS, Chicago, IL, USA).

Results

Expression of Gper in RS

To define *Gper* expression in rat RS, we analysed mRNA using RT-PCR. The analysis revealed that *Gper* mRNA is expressed in this cells (Fig. 1a). This result was confirmed by Western blot analysis, which gave the expected 42-kDa band for Gper protein (Fig. 1b). GC1 mouse spermatogonial cell line was used as positive control (Sirianni *et al.*, 2008). We also performed immunofluorescence staining, which indicated a strong Gper immunoreactivity in the cytoplasm of RS (Fig. 1c).

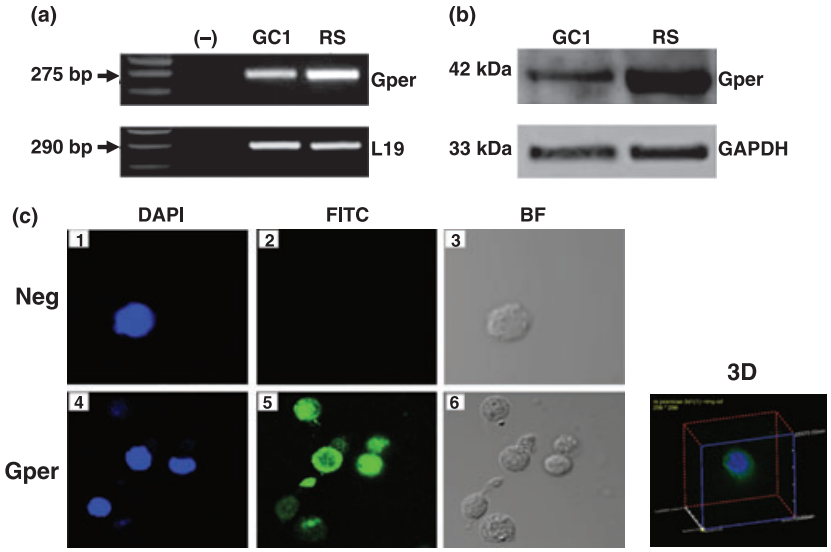

Figure 1 Expression of G-protein-coupled oestrogen receptor 1 (Gper) in round spermatids (RS). (a) Reverse transcription-polymerase chain reaction analysis of Gper in RS. Mouse spermatogonia GC1 (GC1) cells were used as positive control; negative control (–) contained water instead of cDNA. L19 was used as housekeeping gene. Size in base pair of amplified fragments is indicated. (b) Western blot analysis of Gper was performed on 50 μg of total proteins extracted from RS or from GC1 cells. Blots are representative of three independent experiments with similar results. GAPDH was used as loading control. (c) RS were fixed and incubated with polyclonal anti-Gper antibody (1 : 50) followed by incubation with goat anti-rabbit streptavidine-fluoresceine-conjugated secondary antibody as described in 'Materials and methods' (magnification, ×40). Strong immunofluorescence was observed in the cytoplasm of RS (part 5). Negative control slides showed immunonegative reaction for Gper (antibody) in RS (part 2). Nuclei of RS were visualized by DAPI staining (parts 1 and 4). Bright field (BF) contrast is shown (parts 3 and 6). The three-dimensional slice view helps to locate spatially the fluorescent signals in the three-dimensional view (3D).

Expression of ESR1 and ESR2 in RS

We also evaluated ESR1 and ESR2 mRNA and protein expression. RT-PCR analysis demonstrated that RS express both receptor isoforms (Fig. 2a,b). Protein expression analysis, using specific antibodies against the two receptor isoforms, indicated the presence of both ESR1 and ESR2 (Fig. 2c,d), with a lower ESR1 relative expression (Fig. 2e). R2C rat tumour Leydig cell line was used as positive control for both receptor isoforms (Sirianni *et al.*, 2007). As there are controversial reports on ESR1 expression in RS, probably because of specificity of the antibodies used in the studies and other cell types contamination, we performed immunofluorescence analysis for ERS1 in purified RS (Fig. 2f). Our data clearly show expression of ERS1 in our cell population (Fig. 2f).

Oestradiol, G1, PPT and DPN activate ERK1/2 in RS

We investigated the effect of oestradiol (E2) in rat RS on p42/44 MAPK (ERK1/2) phosphorylation. Using increasing doses of E2 (0.001–1 μM) for 30 min, we observed that E2 was able to induce ERK phosphorylation in RS in a dose-dependent manner, with 1 μM dose being the most effective (Fig. 3a). Time course analysis using 1 μM of E2 revealed ERK1/2 activation starting 5 min after treatment with the maximum induction reached at 30 min (Fig. 3b). Treatments with selective activators of ESR1 (PPT) and ESR2 (DPN) at a concentration of 0.1 μM and with specific agonist for Gper (G1) at a concentration of 1 μM were also able to induce ERK1/2 activation (Fig. 3c).

Oestradiol-induced ERK activation is mediated by Gper and ESRs in RS

To verify the involvement of Gper and ESRs in ERK phosphorylation in RS, we tested the effects of inhibitors for EGFR (AG1478; AG), for mitogen-activated ERK-activating protein kinase (MEK; PD98059; PD) and for ER (ICI182780; ICI) on E2- and G1-dependent ERK1/2 activation. We found that in RS, all the tested inhibitors were able to reduce E2- and G1-dependent induction of ERK1/2 significantly (Fig. 4a,b).

Effects of E2, G1, PPT and DPN on cyclin B1 and Bax mRNA expression

To determine the physiological role of oestrogens on RS, we evaluated whether the activation of E2-dependent pathways in RS could lead to a change in the expression of factors regulating cell cycle progression and apoptosis. We first examined changes in *Ccnb1* by quantifying

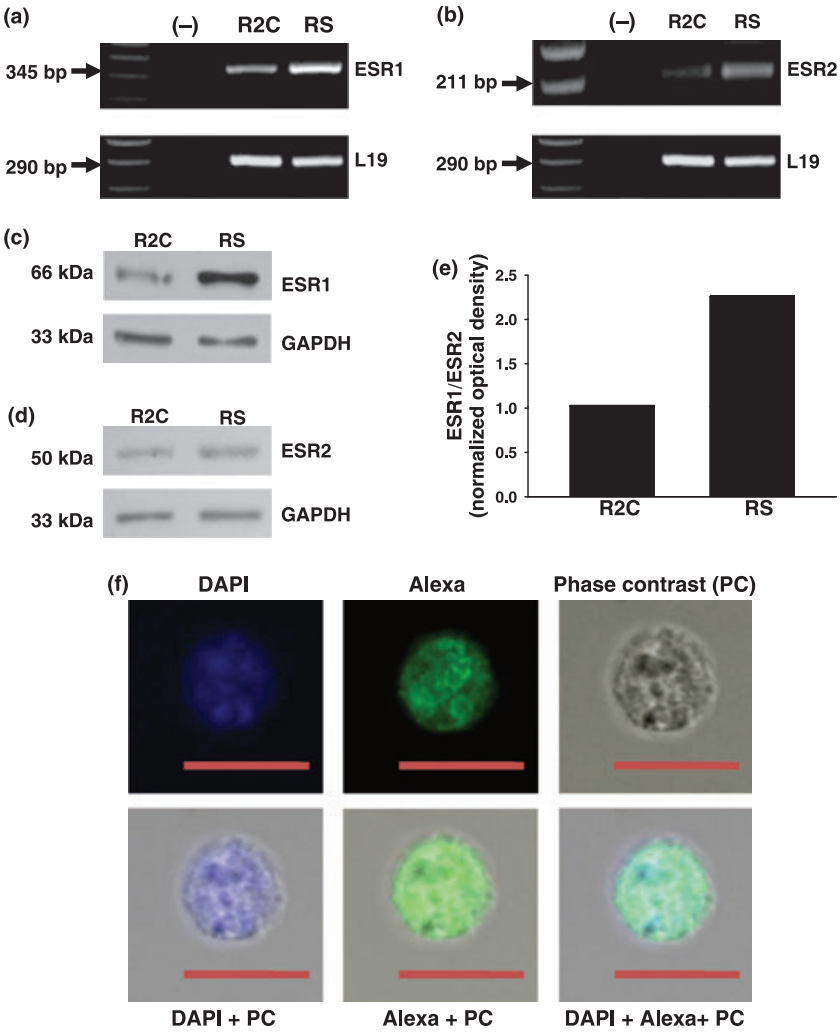

Figure 2 Expression of oestrogen receptor (ESR)1 and ESR2 in round spermatids (RS). Reverse-transcription-polymerase chain reaction (RT-PCR) analysis of ESR1 (a) and ESR2 (b). R2C cells were used as positive control; negative control (–) contained water instead of cDNA. L19 was used as housekeeping gene. Size in base pair of amplified fragments is indicated. Western blot analysis of ESR1 (c) and ESR2 (d) was performed on 50 μg of total proteins extracted from RS or from R2C cells. Blots are representative of three independent experiments with similar results. GAPDH was used as loading control. (e) ESR1 and ESR2 optical densities were normalized to GAPDH optical densities in the same lanes. Normalized values were graphed as ESR1/ESR2 ratio. (f) RS were fixed and incubated with polyclonal anti-ESR1 antibody (1 : 50) followed by incubation with goat anti-rabbit Alexa-F488-conjugated secondary antibody as described in the 'Materials and methods' (magnification, ×60). Strong immunofluorescence was observed mainly in the cytoplasm of RS. Nuclei of RS were visualized by DAPI staining (red line = 10 μm).

mRNA levels (Fig. 5a). Treatments of RS for 6 h with G1 or PPT drastically reduced cyclin B1 mRNA expression; E2 also produced a significant inhibition, but to a lesser extent. Conversely, when cells were treated for the same time with selective agonist of ESR2 (DPN), we found a significant induction of *Ccnb1* mRNA. We also evaluated in the same conditions expression of Bax, the upregulation of which is a feature of germ cell apoptosis in vitro. Treatments of RS with E2, G1 or (PPT) induced Bax mRNA expression (Fig. 5b). The presence of the DPN was able to produce opposite effects determining a decrease in Bax mRNA levels (Fig. 5b).

Discussion

In the present study, we investigated Gper and ESR expression in primary cultures of rat RS and the ability of E2 to activate, through these receptors, rapid response pathways involved in rat RS maturation and apoptosis. Our results clearly show that primary cultures of RS isolated from adult rat testis express ESR1 and ESR2 mRNAs and proteins. Moreover, we also observed that Gper is expressed in the cytoplasm of the same cells.

In mice, it has been claimed that Gper is not involved in oestrogenic responses of the reproductive organs (Otto *et al.*, 2009). This conclusion was reached in a study after generation of Gper-deficient mice. Gper knockout (KO) male and female mice were found fertile; however, although the study missed data on the spermatogenetic process, a careful examination of oestrogenic response was carried out only in uterus and mammary gland. ESR1 KO animals have reduced fertility because of abnormal fluid reabsorption in the efferent ductules (Hess *et al.*, 1997), whereas spermatogenesis, steroidogenesis and fertility are not affected in ESR2 KO animals (Hewitt *et al.*, 2005b). On the contrary, in aromatase knockout (ArKO) mice, the lack of oestrogen production results in an alteration in a complex hormonal balance controlling meiosis progression

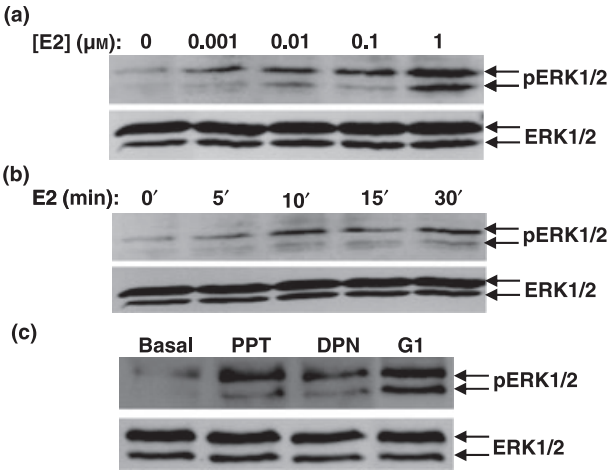

Figure 3 Effects of oestradiol (E2), G1, 4,4′,4″-(4-propyl-[1H]pyrazole-1,3,5-triyl) trisphenol (PPT) and 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) on ERK activation in round spermatids (RS). (a) RS were treated for 30 min with the indicated concentrations of oestradiol (E2). (b) RS were treated for the indicated times with 1 μm of E2. (c) RS were treated for 30 min with PPT (100 nm), DPN (100 nm) or G1 (1 μm). Western blot analyses of pERK1/2 or ERK1/2 were performed on 50 μg of total proteins extracted from RS untreated (basal) or treated as indicated. Blots are representative of three independent experiments with similar results. Protein expression in each lane was normalized to the ERK1/2 content.

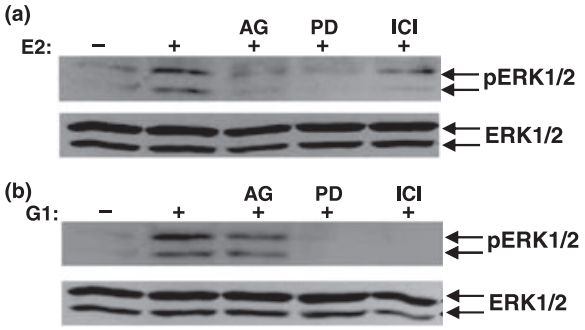

Figure 4 Effects of ERs and G-protein-coupled oestrogen receptor 1 inhibitors on E2- and G1-dependent ERK activation in round spermatids (RS). RS were treated for 30 min with AG1478 (AG; 10 μm) or PD98059 (PD; 10 μm) or ICI182780 (ICI; 10 μm) before being treated for 30 min with E2 (1 μm; a) and G1. (b) Western blot analyses of pERK1/2 or ERK1/2 were performed on 50 μg of total proteins extracted from RS untreated (–) or treated as indicated. Blots are representative of three independent experiments with similar results. Protein expression in each lane was normalized to the ERK1/2 content.

and apoptosis leading to a significant decrease in spermatocytes, round and elongated spermatids numbers (Robertson *et al.*, 1999, 2002). Moreover, recent studies have shown that ligand-independent ESR1 signalling (Sinkevicius *et al.*, 2009) and ERE-independent (non-ERE-dependent) oestrogen pathways play a prominent role in

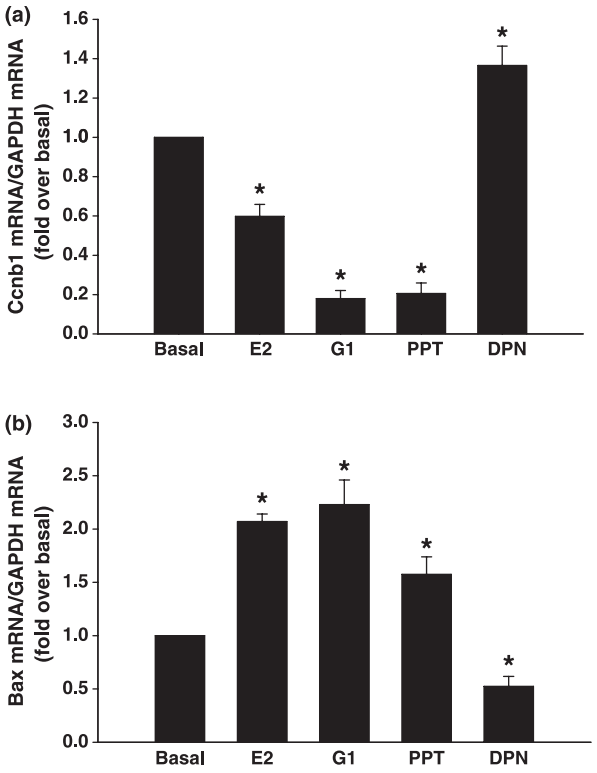

Figure 5 Effects of E2, G1, 4,4′,4″-(4-propyl-[1H]pyrazole-1,3,5-triyl) trisphenol (PPT) and 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) on *Ccnb1* and Bax mRNA expression. Round spermatids (RS) were treated in serum-free medium for 6 h with E2 (1 μm), G1 (1 μm), PPT (100 nm) or DPN (100 nm). Total RNA was extracted; real-time reverse transcription-polymerase chain reaction was used to analyse *Ccnb1* (a) and Bax (b) mRNA levels. Columns indicate mean of values from three separate RNA samples; bars represent standard errors. Each sample was normalized to its GAPDH mRNA content. $*p < 0.001$, compared with basal.

ESR1 action in the testis (Weiss *et al.*, 2008) including pathways that regulate water reabsorption. In contrast, oestrogen-dependent ESR1 signalling seems to be required for germ cell viability (Sinkevicius *et al.*, 2009). These data are confirmed by the more severe testicular phenotype observed in ArKO mice compared with oestrogen receptors knockout (ERKO) mice (Hewitt *et al.*, 2005b). This last observation supports the hypothesis that an alternative receptor could be involved in mediating the effects of oestrogen on spermatogenesis. Moreover, in zebrafish, a functionally active membrane oestrogen receptor that shows great homology to the mammalian Gper has been identified (Liu *et al.*, 2009). This receptor was highly expressed in the brain, and in testicular cells. These recent observations support the idea that investigating the expression and the role of Gper in germ cells is potentially useful to understand well the complex role of oestrogens in regulating germ cell maturation. In previous studies, we

showed that E2, through Gper and ESR1, activates the rapid EGFR/ERK1/2 signalling cascade modulating proliferation in a spermatogonia mouse cell line (Sirianni *et al.*, 2008) and regulating apoptosis of rat pachytene spermatocytes (Chimento *et al.*, 2010). Here, we focused on a more differentiated rat germ cell type, RS, investigating the ability of oestrogens to activate similar pathways potentially involved in RS maturation and/or apoptosis.

ERK1/2 are present in Sertoli cells and in all male rat germ cells and play critical roles in spermatogenesis and sperm functions (Wong & Yan Cheng, 2005; Almog & Naor, 2008; Godet *et al.*, 2008). In this study, we demonstrated that E2 was able to induce rapid ERK1/2 phosphorylation in rat RS. The same effects observed with E2 were also reproduced by the treatment with the Gper-specific agonist G1. The ESR1-specific agonist PPT and the ESR2-specific agonist DPN also induced rapid ERK1/2 phosphorylation. These data show an involvement of ESR1, ESR2 and Gper in ERK1/2 activation. The involvement of Gper pathway was confirmed using EGFR inhibitor AG1478, which was able to block E2- and G1-dependent ERK1/2 phosphorylation. Moreover, the inhibition in the presence of pure ER antagonist (ICI182780) on E2- and G1-dependent ERK1/2 activation demonstrated that in rat RS, Gper and ESR1 could activate ERK1/2 through the same signalling pathway downstream of EGFR as demonstrated in other cell systems (Maggiolini & Picard, 2010). It has been demonstrated by Migliaccio *et al.* (2005) that oestradiol antagonists heavily reduce EGFR tyrosine phosphorylation and the multiple related events including MAPK activation. On the basis of that study and our data, we hypothesize that in our system, E2 can activate a rapid pathway through ESRs determining EGFR tyrosine phosphorylation and then ERK1/2 activation. Another recent study (Lucas *et al.*, 2008) has shown a similar mechanism in Sertoli cells, where E2 activates Src-mediated ER translocation to the plasma membrane causing activation of EGFR/MAPK signalling pathway. In our study, we found that treatments of RS with E2, G1 or PPT rapidly activated ERK1/2, reduced cyclin B1 mRNA and increased Bax mRNA expression.

The link between ERK1/2 activation and modulation of these factors involved in the control of RS maturation/apoptosis, an aspect that is currently under investigation in our lab, remains to be defined. We hypothesize that in rat RS, ERK1/2 activation increases c-Jun N-terminal kinase (JNK) activity, as demonstrated previously in *Xenopus laevis* oocytes (Bagowski *et al.*, 2001), a phenomenon that can determine phosphorylation of AP-1 family members, such as c-jun, regulating expression of cyclin B1 and Bax. Importantly, rat cyclin B1 promoter contains several potential consensus sites where c-jun can exert its action (Trembley *et al.*, 2000), and Bax plays an essential role in JNK-dependent apoptosis (Lei *et al.*, 2002). Our hypothesis is supported by our previous reports showing that E2/ESR1 and/or Gper/ERK1/2 pathways mediate phosphorylation of AP-1 family members such as c-fos in spermatogonia (Sirianni *et al.*, 2008) and c-jun in spermatocytes (Chimento *et al.*, 2010) regulating expression of cyclins or apoptotic factors.

Another intriguing aspect of our data is that the agonist of ESR2, DPN, induces ERK1/2 phosphorylation but determines and increases cyclin B1 and decreases Bax mRNA expressions. This phenomenon can be explained by a different role of ESR2 in mediating the activation of different E2-dependent pathways (Matthews & Gustafsson, 2003).

In macrophages, E2 concomitantly can induce pro-apoptotic and anti-apoptotic signals activating different pathways (Subramanian & Shaha, 2007). Moreover, in breast cancer cell lines, it has been shown for oestrogen-responsive target genes, regulated by an AP-1 dependent mechanism, that activities of AP-1 factors are altered by ESR2 (Matthews *et al.*, 2006). We observed that ESR1 and Gper are involved in a pathway determining the activation of apoptosis, whereas ESR2 mediates an anti-apoptotic signal. Stimulation of both pathways through E2 determines a prevalence of apoptotic signals. This could be explained by the higher ESR1 and Gper expression compared with ESR2. This is further confirmed by the ability of PPT or G1 to activate apoptotic signals, whereas DPN produced opposite effects. Thus, in rat RS, E2 could potentially activate different pathways contributing to the complex balance between cellular maturation and cell death.

In summary, the present study demonstrates, for the first time, that Gper is expressed in rat RS and that oestrogens through Gper and ESRs are able to activate the rapid EGFR/ERK signalling cascade. These data also demonstrate the independent (not mediated by Sertoli cells) capability of rat RS to respond to oestrogens modulating expression of genes controlling rat RS apoptosis and differentiation such as cyclin B1 and Bax.

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References

Almog T & Naor Z. (2008) Mitogen activated protein kinases (MAPKs) as regulators of spermatogenesis and spermatozoa functions. *Mol Cell Endocrinol* 282, 39–44.

Bagowski CP, Xiong W & Ferrell JE Jr. (2001) c-Jun N-terminal kinase activation in *Xenopus laevis* eggs and embryos. A possible non-genomic role for the JNK signaling pathway. *J Biol Chem* 276, 1459–1465.

Bellve AR, Cavicchia JC, Millette CF, O'Brien DA, Bhatnagar YM & Dym M. (1977) Spermatogenic cells of the prepuberal mouse:

isolation and morphological characterization. *J Cell Biol* 74, 68–85.

Bjornstrom L & Sjoberg M. (2005) Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. *Mol Endocrinol* 19, 833–842.

Blanco-Rodriguez J & Martinez-Garcia C. (1997) Apoptosis pattern elicited by oestradiol treatment of the seminiferous epithelium of the adult rat. *Reproduction* 110, 61–70.

Bourguiba S, Genissel C, Lambard S, BouraÇma H & Carreau S. (2003) Regulation of aromatase gene expression in Leydig cells and germ cells. *J Steroid Biochem Mol Biol* 86, 335–343.

Brailoiu E, Dun SL, Brailoiu GC, Mizuo K, Sklar LA, Oprea TI, Prossnitz ER & Dun NJ. (2007) Distribution and characterization of estrogen receptor G protein-coupled receptor 30 in the rat central nervous system. *J Endocrinol* 193, 311–321.

Carreau S & Hess R. (2010) Oestrogens and spermatogenesis. *Philos Trans R Soc B* 365, 1517–1535.

Carreau S, Delalande C, Silandre D, Bourguiba S & Lambard S. (2006) Aromatase and estrogen receptors in male reproduction. *Mol Cell Endocrinol* 246, 65–68.

Chagin AS & Savendahl L. (2007) GPR30 estrogen receptor expression in the growth plate declines as puberty progresses. *J Clin Endocrinol Metab* 92, 4873–4877.

Chapman DL & Wolgemuth DJ. (1993) Isolation of the murine cyclin B2 cDNA and characterization of the lineage and temporal specificity of expression of the B1 and B2 cyclins during oogenesis, spermatogenesis and early embryogenesis. *Development* 118, 229–240.

Chimento A, Sirianni R, Delalande C, Silandre D, Bois C, Andò S, Maggiolini M, Carreau S & Pezzi V. (2010) 17β-estradiol activates rapid signaling pathways involved in rat pachytene spermatocytes apoptosis through GPR30 and ERα. *Mol Cell Endocrinol* 320, 136–144.

D'Souza R, Gill-Sharma MK, Pathak S, Kedia N, Kumar R & Balasinor N. (2005) Effect of high intratesticular estrogen on the seminiferous epithelium in adult male rats. *Mol Cell Endocrinol* 241, 41–48.

Ebling FJP, Brooks AN, Cronin AS, Ford H & Kerr JB. (2000) Estrogenic induction of spermatogenesis in the hypogonadal mouse. *Endocrinology* 141, 2861–2869.

Filardo EJ, Quinn JA, Bland KI & Frackelton AR Jr. (2000) Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol Endocrinol* 14, 1649–1660.

Filardo EJ, Quinn JA, Frackelton AR Jr & Bland KI. (2002) Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. *Mol Endocrinol* 16, 70–84.

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.

Funakoshi T, Yanai A, Shinoda K, Kawano MM & Mizukami Y. (2006) G protein-coupled receptor 30 is an estrogen receptor in the plasma membrane. *Biochem Biophys Res Commun* 346, 904–910.

Gancarczyk M, Paziewska-Hejmej A, Carreau S, Tabarowski Z & Bilinska B. (2004) Dose- and photoperiod-dependent effects of 17beta-estradiol and the anti-estrogen ICI 182,780 on testicular structure, acceleration of spermatogenesis, and aromatase immunoexpression in immature bank voles. *Acta Histochem* 106, 269–278.

Godet M, Thomas A, Rudkin BB & Durand P. (2000) Developmental changes in cyclin B1 and cyclin-dependent kinase 1 (CDK1) levels in the different populations of spermatogenic cells of the post-natal rat testis. *Eur J Cell Biol* 79, 816–823.

Godet M, Sabido O, Gilleron J & Durand P. (2008) Meiotic progression of rat spermatocytes requires mitogen-activated protein kinases of Sertoli cells and close contacts between the germ cells and the Sertoli cells. *Dev Biol* 315, 173–188.

Gromoll J, Wessels J, Rosiepen G, Brinkworth MH & Weinbauer GF. (1997) Expression of mitotic cyclin B1 is not confined to proliferating cells in the rat testis. *Biol Reprod* 57, 1312–1319.

Harrington WR, Kim SH, Funk CC, Madak-Erdogan Z, Schiff R, Katzenellenbogen JA & Katzenellenbogen BS. (2006) Estrogen dendrimer conjugates that preferentially activate extranuclear, nongenomic versus genomic pathways of estrogen action. *Mol Endocrinol* 20, 491–502.

Hess RA & Carnes K. (2004) The role of estrogen in testis and the male reproductive tract: a review and species comparison. *Anim Reprod* 1, 5–30.

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.

Hewitt SC, Deroo BJ & Korach KS. (2005a) Signal transduction. A new mediator for an old hormone? *Science* 307, 1572–1573.

Hewitt SC, Harrell JC & Korach KS. (2005b) Lessons in estrogen biology from knockout and transgenic animals. *Annu Rev Physiol* 67, 285–308.

Isensee J, Meoli L, Zazzu V, Nabzdyk C, Witt H, Soewarto D *et al.* (2009) Expression pattern of G protein-coupled receptor 30 in LacZ reporter mice. *Endocrinology* 150, 1722–1730.

Kaushal N & Bansal MP. (2007) Dietary selenium variation-induced oxidative stress modulates CDC2/cyclin B1 expression and apoptosis of germ cells in mice testis. *J Nutr Biochem* 18, 553–564.

Klinefelter GR, Hall PF & Ewing LL. (1987) Effect of luteinizing hormone deprivation *in situ* on steroidogenesis of rat Leydig cells purified by a multistep procedure. *Biol Reprod* 36, 769–783.

Lei K, Nimnual A, Zong WX, Kennedy NJ, Flavell RA, Thompson CB, Bar-Sagi D & Davis RJ. (2002) The Bax subfamily of Bcl2-related proteins is essential for apoptotic signal transduction by c-Jun NH2-terminal kinase. *Mol Cell Biol* 22, 4929–4942.

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.

Levin ER. (2005) Integration of the extranuclear and nuclear actions of estrogen. *Mol Endocrinol* 19, 1951–1959.

Levin ER & Pietras R. (2008) Estrogen receptors outside the nucleus in breast cancer. *Breast Cancer Res Treat* 108, 351–361.

Liu X, Zhu P, Sham KWY, Yuen JML, Xie C, Zhang Y *et al.* (2009) Identification of a membrane estrogen receptor in zebrafish with homology to mammalian GPER and its high expression in early germ cells of the testis. *Biol Reprod* 80, 1253–1261.

Lucas TFG, Siu ER, Esteves CA, Monteiro HP, Oliveira CA, Porto CS & Lazari MF. (2008) 17beta-estradiol induces the translocation of the estrogen receptors ESR1 and ESR2 to the cell membrane, MAPK3/1 phosphorylation and proliferation of cultured immature rat Sertoli cells. *Biol Reprod* 78, 101–114.

Maggiolini M & Picard D. (2010) The unfolding stories of GPR30, a new membrane-bound estrogen receptor. *J Endocrinol* 204, 105–114.

Maggiolini M, Vivacqua A, Fasanella G, Recchia AG, Sisci D, Pezzi V, Montanaro D, Musti AM, Picard D & Ando S. (2004) The G protein-coupled receptor GPR30 mediates c-fos up-regulation by

17beta-estradiol and phytoestrogens in breast cancer cells. *J Biol Chem* 279, 27008–27016.

Manavathi B & Kumar R. (2006) Steering estrogen signals from the plasma membrane to the nucleus: two sides of the coin. *J Cell Physiol* 207, 594–604.

Matthews J & Gustafsson JA. (2003) Estrogen signaling: a subtle balance between ERa and ERb. *Mol Interv* 3, 281–292.

Matthews J, Wihlen B, Tujague M, Wan J, Strom A & Gustafsson JA. (2006) Estrogen receptor (ER) β modulates ERα-mediated transcriptional activation by altering the recruitment of c-Fos and c-Jun to estrogen-responsive promoters. *Mol Endocrinol* 20, 534–543.

Migliaccio A, Di Domenico M, Castoria G, Nanayakkara M, Lombardi M, de Falco A, Bilancio A, Varricchio L, Ciociola A & Auricchio F. (2005) Steroid receptor regulation of epidermal growth factor signaling through Src in breast and prostate cancer cells: steroid antagonist action. *Cancer Res* 65, 10585–10593.

Mishra DP & Shaha C. (2005) Estrogen-induced spermatogenic cell apoptosis occurs via the mitochondrial pathway: role of superoxide and nitric oxide. *J Biol Chem* 280, 6181–6196.

Mutembei HM, Pesch S, Schuler G & Hoffmann B. (2005) Expression of oestrogen receptors alpha and beta and of aromatase in the testis of immature and mature boars. *Reprod Domest Anim* 40, 228–236.

O'Donnell L, Robertson KM, Jones ME & Simpson ER. (2001) Estrogen and spermatogenesis. *Endocr Rev* 22, 289–318.

Oldereid NB, Angelis PD, Wiger R & Clausen OPF. (2001) Expression of Bcl-2 family proteins and spontaneous apoptosis in normal human testis. *Mol Hum Reprod* 7, 403–408.

Otto C, Fuchs I, Kauselmann G, Kern H, Zevnik B, Andreasen P *et al.* (2009) GPR30 does not mediate estrogenic responses in reproductive organs in mice. *Biol Reprod* 80, 34–41.

Pedram A, Razandi M & Levin ER. (2006) Nature of functional estrogen receptors at the plasma membrane. *Mol Endocrinol* 20, 1996–2009.

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.

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*. *J Clin Endocrinol Metab* 85, 2057–2067.

Porter LA, Cukier IH & Lee JM. (2003) Nuclear localization of cyclin B1 regulates DNA damage-induced apoptosis. *Blood* 101, 1928–1933.

Prossnitz ER & Maggiolini M. (2009) Mechanisms of estrogen signaling and gene expression via GPR30. *Mol Cell Endocrinol* 308, 32–38.

Ramesh R, Pearl CA, At-Taras E, Roser JF & Berger T. (2007) Ontogeny of androgen and estrogen receptor expression in porcine testis: effect of reducing testicular estrogen synthesis. *Anim Reprod Sci* 102, 286–299.

Revankar CM, Cimino DF, Sklar LA, Arterburn JB & Prossnitz ER. (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* 307, 1625–1630.

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.

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.

Sakamoto H, Matsuda KI, Hosokawa K, Nishi M, Morris JF, Prossnitz ER & Kawata M. (2007) Expression of GPR30, a G protein-coupled membrane estrogen receptor, in oxytocin neurons of the rat paraventricular and supraoptic nuclei. *Endocrinology* 148, 5842–5850.

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.

Sierens JE, Sneddon SF, Collins F, Millar MR & Saunders PTK. (2005) Estrogens in testis biology. *Ann NY Acad Sci* 1061, 65–76.

Sinha Hikim AP & Swerdloff RS. (1999) Hormonal and genetic control of germ cell apoptosis in the testis. *Rev Reprod* 4, 38–47.

Sinkevicius KW, Laine M, Lotan TL, Woloszyn K, Richburg JH & Greene GL. (2009) Estrogen-dependent and -independent estrogen receptor-α signaling separately regulate male fertility. *Endocrinology* 150, 2898–2905.

Sirianni R, Chimento A, Malivindi R, Mazzitelli I, Andò S & Pezzi V. (2007) Insulin-like growth factor-I, regulating aromatase expression through steroidogenic factor 1, supports estrogen-dependent tumor Leydig cell proliferation. *Cancer Res* 67, 8368–8377.

Sirianni R, Chimento A, Ruggiero C, De Luca A, Lappano R, Andò S, Maggiolini M & Pezzi V. (2008) The novel estrogen receptor, G protein-coupled receptor 30, mediates the proliferative effects induced by 17β-estradiol on mouse spermatogonial GC-1 cell line. *Endocrinology* 149, 5043–5051.

Sofikitis N, Giotitsas N, Tsounapi P, Baltogiannis D, Giannakis D & Pardalidis N. (2008) Hormonal regulation of spermatogenesis and spermiogenesis. *J Steroid Biochem Mol Biol* 109, 323–330.

Song RXD & Santen RJ. (2006) Membrane initiated estrogen signaling in breast cancer. *Biol Reprod* 75, 9–16.

Song RXD, Zhang Z & Santen RJ. (2005) Estrogen rapid action via protein complex formation involving ERα and Src. *Trends Endocrinol Metab* 16, 347–353.

Subramanian M & Shaha C. (2007) Up-regulation of Bcl-2 through ERK phosphorylation is associated with human macrophage survival in an estrogen microenvironment. *J Immunol* 179, 2330–2338.

Trembley JH, Chen Z, Rodrigues CMP, Kren BT & Steer CJ. (2000) Genomic organization and promoter characterization of the rat cyclin B1 gene. *Gene* 255, 93–104.

Walczak-Jedrzejowska R, Slowikowska-Hilczer J, Marchlewsk K, Oszukowska E & Kula K. (2007) During seminiferous tubule maturation testosterone and synergistic action of FSH with estradiol support germ cell survival while estradiol alone has pro-apoptotic effect. *Folia Histochem Cytobiol* 41, S59–S64.

Wang C, Prossnitz ER & Roy SK. (2007) Expression of G protein-coupled receptor 30 in the hamster ovary: differential regulation by gonadotropins and steroid hormones. *Endocrinology* 148, 4853–4864.

Weiss J, Bernhardt ML, Laronda MM, Hurley LA, Glidewell-Kenney C, Pillai S, Tong M, Korach KS & Jameson JL. (2008) Estrogen actions in the male reproductive system involve estrogen response element-independent pathways. *Endocrinology* 149, 6198–6206.

Wong CH & Yan Cheng C. (2005) Mitogen-activated protein kinases, adherens junction dynamics, and spermatogenesis: a review of recent data. *Dev Biol* 286, 1–15.

Yamashita S. (2004) Localization of estrogen and androgen receptors in male reproductive tissues of mice and rats. *Anat Rec A Discov Mol Cell Evol Biol* 279, 768–778.

Yu Q & Wu J. (2008) Involvement of cyclins in mammalian spermatogenesis. *Mol Cell Biochem* 315, 17–24.

Zhou Q, Nie R, Prins GS, Saunders PTK, Katzenellenbogen BS & Hess RA. (2002) Localization of androgen and estrogen receptors in adult male mouse reproductive tract. *J Androl* 23, 870–881.

Original Research Article

Nandrolone and stanozolol induce Leydig cell tumor proliferation through an estrogen-dependent mechanism involving IGF-I system

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Abstract

Several substances such as anabolic androgenic steroids (AAS), peptide hormones like insulin-like growth factor-I (IGF-I), aromatase inhibitors and estrogen antagonists are offered via the internet, and are assumed without considering the potential deleterious effects that can be caused by their administration. In this study we aimed to determine if nandrolone and stanozolol, two commonly used AAS, could have an effect on Leydig cell tumor proliferation and if their effects could be potentiated by the concomitant use of IGF-I. Using a rat Leydig tumor cell line, R2C cells, as experimental model we found that nandrolone and stanozolol caused a dose-dependent induction of aromatase expression and estradiol (E2) production. When used in combination with IGF-I they were more effective than single molecules in inducing aromatase expression. AAS exhibited estrogenic activity and induced rapid estrogen receptor (ER)-dependent pathways involving IGF1R, AKT and ERK1/2 phosphorylation. Inhibitors for these kinases decreased AAS-dependent aromatase expression. Up-regulated aromatase levels and related E2 production increased cell proliferation as a consequence of increased cyclin E expression. The observation that ER antagonist ICI182,780 was also able to significantly reduce ASS- and AAS+IGF-induced cell proliferation, confirmed a role for estrogens in AAS-dependent proliferative effects. Taken together these data clearly indicate that the use of high doses of AAS, as it occurs in doping practice, enhances Leydig cell proliferation, increasing the risk of tumor development. This risk is higher when AAS are used in association with IGF-I. To our knowledge this is the first report directly associating AAS and testicular cancer.

Introduction

Substance abuse has become increasingly widespread among athletes at sub competitive and recreational level, raising concern for human health. In addition to the illicit use of substances to increase performance of athletes and to enhance the muscular mass and strength, more recently the use of some agents has been extended to non-athletes with the aim to combat ageing, obesity and improve appearance or libido (Calfee and Fadale, 2006). Since its discovery in 1935, numerous derivatives of testosterone have been synthesized, with the goals of prolonging its biological activity in vivo, producing orally active androgens, and developing products, commonly referred to as anabolic androgenic steroids (AAS), that are more anabolic and less androgenic than the parent molecule. AAS doping is undeniably rampant worldwide. The doses of testosterone or other androgens used by athletes are substantially larger than those prescribed for the treatment of androgen deficiency. In one survey (Evans, 1997a), 50% of androgen users reported using at least 500 mg of testosterone weekly or an equivalent dose of another androgen; in another survey (Pope and Katz, 1994), almost one fourth of androgen users assumed 1000 mg of testosterone weekly or an equivalent dose of other androgens. It is becoming increasingly clear that the abuse of AAS is associated with serious adverse effects to the liver (Wakabayashi et al., 1984) and the cardiovascular (Fineschi et al., 2001; Fineschi et al., 2007) central nervous (Shahidi, 2001), musculoskeletal (Al-Ismail et al., 2002), endocrine (Shahidi, 2001) and reproductive (Dohle et al., 2003; Eklöf et al., 2003) systems. As a consequence of their effects on the endocrine and reproductive systems AAS cause suppressed spermatogenesis, gynecomastia and virilization. Clinical reports highlight a link between AAS abuse and various types of cancer, mainly to the liver such as hepatocellular adenomas and adenocarcinomas (Socas et al., 2005), however other types of cancer such as Wilms' tumors have been reported (Bronson and Matherne, 1997; Modlinski and Fields, 2006).

Androgens exert their biological effect through an intracellular receptor, the androgen receptor (AR), that is present in the reproductive tract as well as in many non-reproductive tissues, including bone, skeletal muscle, brain, liver, kidney and adipocytes. Binding of androgens to AR determines receptor dimerization, nuclear translocation and binding to specific responsive elements (ARE) present in the promoter region of target genes (Li and Al-Azzawi, 2009). Androgens mechanism of action in skeletal muscle cells is well documented and includes up-regulation of markers of myogenic differentiation, such as MyoD and myosin heavy chain II (Singh et al., 2003; Bhasin et al., 2003; Singh et al., 2006). However, androgens can be converted to estrogens through the action of the aromatase enzyme. In the human, aromatase is expressed in a number of cells including

brain, skin fibroblasts, bone, adipose tissue, in steroidogenic tissues such as placenta and gonads (Simpson et al., 1994), in particular in man aromatase is present in most of the testicular cells. Estrogens are required for a normal spermatogenesis, which seems extremely sensitive to estrogen concentration. Transgenic mice lacking aromatase expression (ArKO mice) show an age-dependent disruption of spermatogenesis, a significant reduction in testis weight and compromised fertility (Honda et al., 1998; Robertson et al., 2002). Similarly, men with inactivating mutations of the aromatase gene, leading to the lack of the estrogen synthesis, are infertile (Faustini-Fustini et al., 1999). On the other hand, about half of the male transgenic mice over-expressing aromatase and presenting enhancement of circulating 17β-estradiol (E2) levels are infertile and/or have enlarged testis and show Leydig cell hyperplasia and Leydig cell tumors (Fowler et al., 2000). In a previous study we have shown that Leydig cell tumor is characterized by aromatase overexpression and consequent increased estrogen production, that contributes to inducing tumor cell proliferation (Sirianni et al., 2007).

Testosterone, nandrolone, stanozolol, methandienone, and methenolol are the most frequently abused androgens (Pope and Katz, 1994; Evans, 1997a; Evans, 1997b). These androgens can be differentially metabolized by aromatase, specifically nandrolone can be converted to estrogens, while stanozolol is a non-aromatizable androgen.

In addition to the use of androgens, athletes also abuse other drugs to purportedly enhance muscle building, muscle shaping or athletic performance (Evans, 1997a). These accessory drugs include stimulants, such as amphetamine, clenbuterol, ephedrine, and thyroxine, anabolic agents such as growth hormone (GH), insulin and insulin-like growth factor-I (IGF-I) and drugs perceived to reduce adverse effects such as human chorionic gonadotropin (hCG), aromatase inhibitors or estrogen antagonists (Evans, 1997a). In particular, IGF-I, which is the main effector for the action of GH, is a peptide physiologically produced by the liver. The potential benefits of IGF-I administration include increased muscle protein synthesis and the sparing of glycogenolysis with glycogen synthesis and increased fatty acid availability. IGF-I is known to have a role in testicular growth and development and in the control of Leydig cell number (Saez, 1994). IGF-I is produced locally in the testis, in Sertoli, Leydig and peritubular cells derived from the immature testis and cultured in vitro (Casella et al., 1987; Zhou and Bondy, 1993). The crucial role of IGF-I in the development and function of Leydig cells was highlighted by studies on IGF-I gene knockout mice (Liu et al., 1993; Baker et al., 1996). The failure of adult Leydig cells to mature and the reduced capacity for testosterone production in IGF-I knock out (KO) mice are caused by deregulated expression of testosterone biosynthetic and metabolizing enzymes (Wang et al., 2003), expression

levels of all mRNA species associated with testosterone biosynthesis are lower in the absence of IGF-I. Furthermore, IGF-I plays a central role in inducing aromatase expression in Leydig tumor cells, consequently IGF-I increases estrogen production that contributes to the induction of tumor cell proliferation (Sirianni et al., 2007).

Taking into account that Leydig cell tumors are common in young men, the same age group commonly abusing AAS, we wanted to investigate the effects of AAS and IGF-I on Leydig cell tumors. Our hypothesis is that AAS can induce Leydig cell tumor proliferation and that this effect could be potentiated by the concomitant use of IGF-I. To verify this hypothesis in the present study we evaluated on Leydig R2C cells the effects of commonly used ASS, differentially metabolized by aromatase, such as nandrolone (aromatizable) and stanozolol (non-aromatizable), used alone or in association with IGF-I, on aromatase expression and Leydig cell tumor proliferation.

MATERIALS AND METHODS

Cell cultures.

Cells were bought from American Type Culture Collection (LGC Standards, Teddington, Middlesex UK), grown for 2 weeks (four passages) before freezing aliquots. Each aliquot was used for no more than ten passages. R2C cells (a rat Leydig tumor cell line) were cultured in Ham/F-10 (Sigma St Louis, MO, USA) medium supplemented with 15% horse serum (HS), 2.5% fetal bovine serum (FBS) and antibiotics (Invitrogen, S.R.L., San Giuliano Milanese, Italy). Cell monolayers were subcultured onto 30 mm dishes for protein or RNA extraction ($1x10^6$ cells/plate), 12 well culture plates for steroid measurement ($2x10^5$ cells/well) and 24 well culture plates proliferation assay ($1x10^5$ cells/well), and used for experiments 48 h later.

Human embryonic kidney (HEK)-293 cells were cultured in Dulbecco's modified Eagle's/Ham F12 (DME/F12) medium (GIBCO BRL) supplemented with 5% FBS (Invitrogen) and antibiotics. Cell monolayers were subcultured onto 24 well culture plates for transfection experiments.

Cell cultures were treated for the indicated times with PD98059, LY294002, GF109203X (Calbiochem, VWR International S.R.L. Milano), AG1024, ICI182,780 nandrolone, Stanozolol, IGF-I, 17β-estradiol, testosterone and dihydrotestosterone (Sigma St Louis, MO, USA).

Radioimmunoassay.

Prior to experiments, R2C cells were maintained in Ham/F-10 without serum or antibiotics (serum-free medium). Cells were then treated as necessary and estradiol content of medium recovered from each well was determined against standards prepared in serum-free medium using a radioimmunoassay kit (Diagnostic System Laboratories, Webster, TX, USA). Results were normalized to the cellular protein content per well. For IGF-I determination cells were cultured for 48h before treatment for an additional 72h in Ham/F-10 containing 1 % dextrane charcoal coated (DCC) FBS. IGF-I content in medium recovered from each well of R2C cells was determined following extraction and assay protocol provided with the rat IGF-I radioimmunoassay kit (DSL 2900; Diagnostic System Laboratories, Webster, TX, USA).

Aromatase activity assay.

The aromatase activity in sub-confluent 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 as previously shown (Lephart and Simpson, 1991; Sirianni et al., 2009).

Western blot analysis.

Methods for protein extraction and blots preparation have been previously published (Sirianni et al., 2007). Blots were incubated overnight at 4°C with (a) anti-human P450 aromatase antibody (Serotec, Oxford, UK) (1:50), (b) anti-cyclin E (M-20) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:1000), (c) anti-pIGF1R antibody (1:500; Cell Signaling Technology, Massachusetts, USA), (d) anti-pERK antibody (1:500; Cell Signaling Technology, Massachusetts, USA), (e) anti-pAKT antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), (f) anti-IGF1R antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), (g) anti-ERK antibody (1:1000; Cell Signaling Technology, Massachusetts, USA), (h) anti-AKT antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), (i) anti-GAPDH antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:1000). Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ, USA) 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 GAPDH antibody.

RNA extraction, reverse transcription and PCR.

The TRizol RNA isolation system (Invitrogen S.r.l., San Giuliano Milanese (MI), Italy) was used to extract RNA from R2C cells. Each RNA sample was treated with DNase I (Ambion, Austin, TX, USA), 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 transcriptase system kit (Promega Italia S.r.l., Milano, Italy). Samples were aliquoted and stored at −20 ◦C. PCR amplification was performed using 1.5 U of Taq DNA polymerase (Promega Italia S.r.l., Milano, Italy) in PCR buffer containing 200 µM dNTP, 1.5mM MgCl2, and 25 pmoles of each primer in a total volume of 50 µl. Primers sequence for the rat aromatase gene, PCR conditions and number of cycles were previously published (Catalano et al., 2010). L19 ribosomal protein mRNA was used as housekeeping gene, PCR conditions, number of cycles and primers sequence were previously published (Chimento et al., 2010). To avoid products due to DNA contamination, primers were designed to amplify a region across different exons. PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide (Sigma–Aldrich Italia S.r.l., Milano, Italy).

Assessment of cell proliferation.

3-[4,5-Dimethylthiaoly]-2,5-diphenyltetrazolium bromide (MTT) assay was conducted to detect cell proliferation (Sylvester, 2011). A total of $1x10^5$ cells were seeded onto twenty-four-well plates in complete medium and let grow for 2 days. Prior to experiments, cells were maintained overnight in Ham/F-10 serum-free medium and the day after treated. There were triplicates for each concentration. Forty-eight hours after treatment fresh MTT re-suspended in PBS was added to each well (final concentration 0.33 mg/ml). After 1h incubation, the culture media were discarded and replaced with 100 µl of DMSO. The optical density was measured at 570 nm in a spectrophotometer.

Transfection assay.

Before transfection, complete medium was removed, and 0.5 ml of DMEM/F12 without phenol red, serum or antibiotics was added to the plates. Transfection was performed using Fugene6 reagent (Roche Diagnostics, Mannheim, Germany), following the manufacturer's instruction. Plasmids were used at the concentration of 0.5 µg/well for the XETL (Bunone et al., 1996) promoter-luciferase reporter plasmid, of 0.1 µg/well for ERα expression vector (Tora el al., 1986), of 10 ng/well for the β-galactosidase control vector (Promega Italia S.r.l., Milano, Italy). Four hours after transfection, the medium was removed and replaced with DMEM/F12 without phenol red, serum or antibiotics and supplemented with the indicated concentrations of treatments for 24 h. Cells were lysed using the passive lysis buffer (Promega Italia S.r.l., Milano, Italy), and enzymatic activities were assayed using the Luciferase (Promega Italia S.r.l., Milano, Italy) and β-galactosidase (Ambion, Austin, TX, USA) assay systems following the manufacturer's instructions. Firefly luciferase values of each sample were normalized by β-galactosidase activity and data were reported as relative light units (RLU) values.

Statistical analysis.

All experiments were conducted at least three times and results were from representative experiments. Data were expressed as mean values ± standard deviation (S.D.) and statistical significance was analysed by Student-Newman-Keuls multiple comparison methods, using SigmaStat version 3.0 (SPSS, Chicago, IL).

RESULTS

Nandrolone and stanozolol control Leydig cell proliferation through the induction of aromatase expression and estradiol production.

We first evaluated if nandrolone and stanozolol were able to induce R2C cell proliferation. Cell proliferation was measured in R2C cells treated with increasing doses of nandrolone (Fig. 1A) and stanozolol (Fig. 1B), revealing a significant induction with all tested doses of both androgens, with 1 μM being the most effective. Since proliferation of R2C cells depends on estrogen production (Sirianni et al., 2007), we evaluated the effects of nandrolone and stanozolol on aromatase expression. For this purpose R2C cells were treated for 24h with increasing doses of the two androgens and aromatase protein expression was evaluated by western blot analysis (Figure 1C, D). Both androgens were able to significantly increase aromatase levels, with maximum induction seen with 1 μM (Fig. 1C, D). We also evaluated endogenous estrogen production in response to nandrolone and stanozolol, revealing the ability of both androgens to increase estradiol production as a consequence of effects on aromatase expression (Fig. 1E). To confirm that proliferative effects depended on the ability of the two androgens to induce estradiol production, cells were treated with nandrolone and stanozolol in the presence of estrogen receptor antagonist ICI182,780 (ICI). As seen in figure 1F ICI reduced the effects of both androgens.

Nandrolone or stanozolol in combination with IGF-I further induce Leydig cell proliferation and aromatase expression.

Since we have previously shown a role for IGF-I in Leydig cell proliferation (Sirianni et al., 2007), we also evaluated effects of combined treatments of IGF-I with nandrolone or stanozolol on cell proliferation in R2C cells (Fig. 2). The dose of 1 μM was used in combination with IGF-I (100 ng/ml) and cell proliferation was measured (Fig. 2A). Results obtained show that both nandrolone and stanozolol have an additive effect with IGF-I in inducing proliferation (Fig. 2A). Also in this case, the estrogen antagonist ICI was able to significantly reduce both AAS- and AAS+IGF-I-dependent proliferative effects (Fig. 2A). An additive effect in inducing aromatase expression was found when IGF was combined with nandrolone or stanozolol (Fig. 2B, C). Data obtained on aromatase expression were also mirrored by effects on aromatase activity (Fig. 2D).

IGF-I receptor (IGF1R) activation by direct ligand binding or by indirect transactivation determines activation of three major transductional pathways: Ras/Raf/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/AKT, and phospholipase C (PLC)/protein kinase C (PKC). We tested the effects on cell proliferation of specific inhibitors such as PD98059 for

MAPK; LY294002 for PI3K/AKT, (LY), GF109203X (GFX) for PLC/PKC and AG1024 (AG) for IGF1R, used in combination with nandrolone or stanozolol (Fig. 3A). The use of AG, LY and GFX was able to reverse nandrolone and stanozolol-induced cell proliferation (Fig. 3A). PD was the only inhibitor that did not interfere with cell proliferation. To demonstrate the link between cell proliferation and estrogen production we evaluated the effects of the inhibitors on aromatase expression (Fig. 3B-D). AG, LY and GFX were able to reduce nandrolone- and stanozolol-induced aromatase mRNA (Fig. 3B) and protein (Fig. 3C, D) levels.

The ability of inhibitors for IGF-I-dependent pathway to control nandrolone and stanozolol effects led us to suppose that the two AAS could regulate IGF-I production in R2C cells. Basal IGF-I content in R2C cell culture medium was 80 mg/ml/mg protein and was increased by 2- and 3.4-fold by nandrolone and stanozolol, respectively (Fig. 4A). When IGF-I was neutralized with a specific antibody cell proliferation was markedly decreased (Fig. 4B). Nandrolone and stanozolol could not overcome the inhibitory effect produced by the antibody (Fig. 4B). These data confirm that R2C proliferation is tightly dependent on IGF-I and indicate that a mechanism through which nandrolone and stanozolol control R2C proliferation is through the induction of IGF-I production.

Nandrolone and stanozolol activate rapid signalling through ERα transactivation.

Recently it has been demonstrated that androgens can activate alternative pathways, in addition to classic genomic mechanisms of regulation of gene expression (Foradori et al., 2008). For this reason we decided to investigate the ability of nandrolone and stanozolol to activate rapid signalling involving IGF1R phosphorylation and activation of kinases involved in cell proliferation. R2C cells were treated for 10 minutes with increasing doses of the two androgens and IGF1R, ERK and AKT phosphorylation was evaluated by western blot analysis. Both androgens were capable to increase kinase activity at all used doses (Fig. 5A, B).

The effects produced by the two AAS on the activation of IGF1R-dependent pathways were reproduced also by E2, dihydrotestosterone (DHT) and testosterone (T) (Fig. 5C). In addition the presence of specific inhibitors for IGF1R, ERK1/2 and AKT were able to prevent kinases activation (Fig. 5C).

There are numerous reports suggesting that androgens might act through ERα by themselves (Maggiolini et al., 1999) and our data shown in Figure 1F and 2A indicate that this could also be the case for the two AAS in R2C cells. To define if the rapid AAS-dependent signaling was mediated by ER, R2C cells were treated with the two androgens in the presence of the estrogen receptor

antagonist ICI182,780. As shown in Figure 5D ICI administration reduced the effect of both androgens on IGF1R phosphorylation.

To evaluate the specificity of nandrolone and stanozolol to transactivate ERα we performed co-transfection experiments using a luciferase reporter plasmid (XETL), a construct containing an estrogen-responsive element (Bunone et al., 1996), and the human ERα expression vector (HEGO) (Tora el al., 1986). To avoid interference by endogenous estrogen receptors transfections were performed in HEK293 cells that do not express ERs. Eighteen hours after transfection cells were exposed to increasing concentrations of the two androgens. Our results demonstrate the ability of both androgens to transactivate ERα (Fig. 5E).

Nandrolone and stanozolol influence cell proliferation by increasing cyclin E expression levels.

To explain the observed effects on cell proliferation we evaluated expression of cyclin E, that we have previously shown to be up-regulated by IGF-I in R2C cells (Sirianni et al., 2007). Cyclin E protein expression was increased by all used doses of both nandrolone (Fig. 6A) and stanozolol (Fig. 6B). In addition, combined treatments with IGF-I and either steroid, caused a stronger induction of cyclin E expression (Fig. 6C, D).

DISCUSSION

Leydig cells are the main site of testosterone biosynthesis in the male mammals, as well as a site of conversion of testosterone to estradiol through aromatase activity (Carreau et al., 2006). Alterations in local estrogen synthesis may have significant consequences in malignancy of these cells. In a previous study, we observed that R2C tumor Leydig cells release a conspicuous amount of E2, significantly higher than normal Leydig cell cultures, as a consequence of aromatase over-expression in tumor cells (Sirianni et al., 2007). It can be suggested that in the presence of increased androgens availability, their metabolism through aromatase, expressed in Leydig cells, increases local estrogen levels, contributing to the initiation or progression of Leydig cell tumor. We tested the effects of two commonly used AAS nandrolone (aromatizable) and stanozolol (non-aromatizable) and evaluated the effects on aromatase expression and Leydig cell proliferation. Since muscle mass and strength are correlated with the administered dose and the circulating concentrations of AAS (Bhasin et al., 2001; Bhasin et al., 2005), the doses commonly used in practice are extremely high and range from 500 to 1000 mg/weekly (Pope and Katz, 1994; Evans, 1997a). These observations support our decision to test the effects of high doses (0.1 to 10 μM) of nandrolone and stanozolol. When we evaluated the effects of these two androgens on aromatase expression we found that they are both able to increase the enzyme protein levels and consequently estrogen production in R2C cells, with stanozolol being more effective in inducing the enzyme but nandrolone more effective in increasing estrogen levels. A plausible explanation for this behavior is that nandrolone is immediately metabolized to estradiol by R2C cells, where aromatase is constitutively active, and the amount left to activate the mechanism responsible for increasing aromatase transcription is less than the concentration effectively used to treat cells. On the other side, stanozolol is not metabolized by aromatase, and the concentration used to treat cells is effectively bio-available to determine the effects on aromatase transcription. Thus, both AAS contribute to estrogen production determining an effect on tumor cell proliferation that we could define “indirect”, not related to their androgenic properties, but estrogen-dependent. This effect is confirmed by the observation that ICI reduces AAS-dependent cell proliferation. However, since we found that treatments with inhibitors for IGF1R, PKC and PI3K were able to reduce AAS effects on aromatase expression and cell proliferation, it could also be suggested that these molecules directly activate non-genomic hormonal signals. We supposed that the two AAS could activate estrogen receptor (ER)-dependent pathways. The estrogenic actions of androgens are established (Rochefort and Garcia, 1983), and the possibility for AAS to activate ERs is consistent, also in our experimental system, with the ability of ICI to reduce AAS-dependent cell proliferation. In

addition, AAS acting on a membrane bound ER activate IGF1R and downstream kinases, as seen in several cell systems (Song et al., 2006). These events could partially explain the inhibition produced by AG1024, the IGF1R inhibitor, and by downstream IGF-I signaling inhibitors (GFX for PKC and LY for PI3K). The use of high doses of AAS could cause in our cell system ER/AR desensitization, explaining the reduced effects seen with 10 μM of both nandrolone and stanozolol. Our results give interesting indications and open several hypothesis on possible non genomic molecular mechanisms induced by AAS involving IGF-I dependent signaling pathways. For example, GPR30, a novel estrogen receptor (Prossnitz and Maggiolini, 2009), could also be activated by AAS, explaining why ICI was not able to completely block nandrolone- and stanozolol-dependent IGF1R activation. Additionally, the two AAS can also work through a membrane AR to activate IGF1R signaling.

It was shown for human primary prostatic stromal cell cultures, that administration of DHT and T, but not of E2, modulated IGF-I protein expression (Le et al., 2006). In our cell model the two androgens induce IGF-I production, and when this growth factor was immunoneutralized, cell proliferation was deeply reduced and nandrolone and stanozolol lost their ability to increase cell proliferation, confirming our previous report indicating IGF-I as indispensable for R2C cell proliferation (Sirianni et al., 2007). In addition, the observation that stanozolol was more effective than nandrolone in inducing IGF-I production, supports the hypothesis that nandrolone metabolism to estradiol reduces its bio-available amount.

IGF-I signalling is highly involved in cancer development and progression (Larsson et al., 2005) exerting powerful effects on each of the key stages of cancer formation: cellular proliferation, apoptosis, angiogenesis, metastasis and resistance to chemotherapeutic agents. We previously showed that IGF-I, endogenously produced by Leydig tumor cells, activating PI3K/AKT and PLC/PKC (but not MAPK) pathways determines an increase in aromatase expression and estrogen production inducing cell proliferation (Sirianni et al., 2007). In the current study we confirmed these data and tested also the effect of combined treatments of nandrolone or stanozolol with IGF-I. Effects of the two AAS were potentiated by the presence of IGF-I. The evaluation of aromatase activity revealed that the combined treatments of nandrolone or stanozolol with IGF-I increased the enzyme activity to levels above those seen with the single treatments. All the effects seen on aromatase were mirrored by changes in R2C proliferative behavior; nandrolone and stanozolol increase cell proliferation and their effects are additive with IGF-I. One mechanism through which estrogens and IGF-I induce cell proliferation is by increasing protein levels of G1 regulatory cyclins A, B1, D1, D3, and E in target cells (Prall et al., 1997; Ma et al., 2009). In our study, we showed that the expression of one of the most important regulators of Leydig cell cycle, cyclin E, can be

increased by nandrolone and stanozolol and significantly increased to a greater extent by the combined treatment with IGF-I. These result further confirm that AAS could contribute to activate expression of estrogen- and IGF-I-dependent genes involved in cell cycle progression.

In conclusion, despite the growing body of data over the past decade that has established that androgens increase muscle mass (Bhasin et al., 2001) in correlation with the administered dose and the circulating concentrations (Bhasin et al., 2005), it should be seriously taken into account the potential dangerous effects produced by the use of AAS on the activation of pathways involved in the progression of a type of cancer, such as Leydig cell tumor, with high incidence in young people, the same age people abusing AAS. In this study we have shown that aromatizable and non-aromatizable androgens can promote testicular tumor development through the induction of aromatase expression, estrogen and IGF-I production. In addition, the two tested androgens binding ERα transactivated IGF1R activating PI3K and PKC pathways determining an induction of R2C tumor cell proliferation. The examined AAS effects are potentiated by the concomitant use of IGF-I. It would be interesting to determine the effect of the two AAS in human cultures of Leydig cells, that, however are currently not available, as well as *in vivo* in an animal model. Before beginning any illegal and self-determined use of doping agents deep consideration should be given to the deleterious effects associated with their use, among which we can now include Leydig cell tumor.

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REFERENCES

Al-Ismail K, Torreggiani W, Munk P, Nicolaou S. 2002. Gluteal mass in a bodybuilder: radiological depiction of a complication of anabolic steroid use. Eur Radiol 12:1366-1369.

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.

Bhasin S, Woodhouse L, Casaburi R, Singh AB, Bhasin D, Berman N, Chen X, Yarasheski KE, Magliano L, Dzekov C, Dzekov J, Bross R, Phillips J, Sinha-Hikim I, Shen R, Storer TW. 2001. Testosterone dose-response relationships in healthy young men. Am J Physiol-Endocrinol Metab 281:E1172-E1181.

Bhasin S, Taylor W, Singh R, Artaza J, Sinha-Hikim I, Jasuja R, Choi H, Gonzalez-Cadavid N. 2003. The mechanisms of androgen effects on body composition: mesenchymal pluripotent cell as the target of androgen action. J Gerontol Series A Biol Sci Med Sci 58:M1103-M1110.

Bhasin S, Woodhouse L, Casaburi R, Singh AB, Mac RP, Lee M, Yarasheski KE, Sinha-Hikim I, Dzekov C, Dzekov J, Magliano L, Storer TW. 2005. Older men are as responsive as young men to the anabolic effects of graded doses of testosterone on the skeletal muscle. J Clin Endocrinol Metab 90:678-688.

Bronson FH, Matherne CM. 1997. Exposure to anabolic-androgenic steroids shortens life span of male mice. Med Sci Sports Exerc 29:615-619.

Bunone G, Briand P, Miksicek R, Picard D. 1996. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J 15:2174-2183

Calfee R, Fadale P. 2006. Popular ergogenic drugs and supplements in young athletes. Pediatrics 117:e577-589.

Carreau S, Delalande C, Silandre D, Bourguiba S, Lambard S. 2006. Aromatase and estrogen receptors in male reproduction. Mol Cell Endocrinol 246:65-8.

Casella SJ, Smith EP, van Wyk JJ, Joseph DR, Hynes MA, Hoyt EC, Lund PK. 1987. Isolation of rat testis cDNAs encoding an insulin-like growth factor I precursor. DNA 6:325-330.

Catalano S, Malivindi R, Giordano C, Gu G, Panza S, Bonofiglio D, Lanzino M, Sisci D, Panno ML, Andò S. 2010. Farnesoid X receptor, through the binding with steroidogenic factor 1-responsive element, inhibits aromatase expression in tumor leydig cells. J Biol Chem 285:5581-5593.

Chimento A, Sirianni R, Delalande C, Silandre D, Bois C, Andò S, Maggiolini M, Carreau S, Pezzi V. 2010. 17β-estradiol activates rapid signaling pathways involved in rat pachytene spermatocytes apoptosis through GPR30 and ERα. Mol Cell Endocrinol. 320:136-44.

Dohle GR, Smit M, Weber RF. 2003. Androgens and male fertility. World J Urol 21:341-345.

Eklöf AC, Thurelius AM, Garle M, Rane A, Sjöqvist F. 2003. The anti-doping hot-line, a means to capture the abuse of doping agents in the Swedish society and a new service function in clinical pharmacology. Eur J Clin Pharmacol 59:571-577.

Evans NA. 1997a. Gym and tonic: a profile of 100 male steroid users. Brit J Sport Med 31:54-58.

Evans NA. 1997b. Local complications of self administered anabolic steroid injections. Brit J Sport Med 31:349-350.

Faustini-Fustini M, RochiraV, Carani C. 1999. Oestrogen deficiency in men: where are we today? Eur J Endocr 140:111-129.

Fineschi V, Baroldi G, Monciotti F, Reattelli LP, Turillazzi E. 2001. Anabolic steroid abuse and cardiac sudden death. Arch Pathol Lab Med 125:253-255.

Fineschi V, Riezzo I, Centini F, Silingardi E, Licata M, Beduschi G, Karch S. 2007. Sudden cardiac death during anabolic steroid abuse: morphologic and toxicologic findings in two fatal cases of bodybuilders. Int J Leg Med 121:48-53.

Foradori CD, Weiser MJ, Handa RJ. 2008. Non-genomic actions of androgens. Front Neuroendocrinol 29:169-181

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 testicular cancer. Am J Pathol 156:347-353.

Honda SI, Harada N, Ito S, Takagi Y, Maeda S. 1998. Disruption of sexual behavior in male aromatase-deficient mice lacking exons 1 and 2 of the cyp19 gene. Biochem Biophys Res Commun 252:445-449.

Larsson O, Girnita A, Girnita L. 2005. Role of insulin-like growth factor 1 receptor signalling in cancer. Brit J Cancer 92:2097-2101.

Le H, Arnold JT, McFAnn KK, Blackman MR. 2006. DHT and testosterone, but not DHEA or E2, differentially modulate IGF-I, IGFBP-2, and IGFBP-3 in human prostatic stromal cells. Am J Physiol Endocrinol Metab 290:E952-960

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

Li J, Al-Azzawi F. 2009. Mechanism of androgen receptor action. Maturitas 63:142-148.

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.

Ma QL, Yang TL, Yin JY, Peng ZY, Yu M, Liu ZQ, Chen FP. 2009. Role of insulin-like growth factor-1 (IGF-1) in regulating cell cycle progression Biochem Biophys Res Commun 389:150-155.

Maggiolini M, Donzè O, Jeannin E, Andò S, Picard D. 1999. Adrenal androgens stimulate the proliferation of breast cancer cells as direct activators of Estrogen Receptor α. Cancer Res 59(19):4864-4869

Modlinski R, Fields K. 2006. The effect of anabolic steroids on the gastrointestinal system, kidneys, and adrenal glands. Curr Sports Med Rep 5(2):104-109.

Pope H & Katz D. 1994. Psychiatric and medical effects of anabolic-androgenic steroid use: a controlled study of 160 athletes. Arch Gen Psychiat 51:375-382.

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.

Prossnitz ER, Maggiolini M. 2009. Mechanisms of estrogen signaling and gene expression via GPR30. Mol Cell Endocrinol 308 (1-2):32-38

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. Endocrinol 143:2913-2921.

Rochefort H, Garcia M. 1983. The estrogenic and antiestrogenic activities of androgens in female target tissues. Pharmacol Ther 23:193-216.

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

Shahidi NT. 2001. A review of the chemistry, biological action, and clinical applications of anabolic-androgenic steroids. Clin Ther 23:1355-1390.

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.

Singh R, Artaza JN, Taylor WE, Gonzalez-Cadavid NF, Bhasin S. 2003. Androgens stimulate myogenic differentiation and inhibit adipogenesis in C3H 10T1/2 pluripotent cells through an androgen receptor-mediated pathway. Endocrinol 144:5081-5088.

Singh R, Artaza JN, Taylor WE, Braga M, Yuan X, Gonzalez-Cadavid NF, Bhasin S. 2006. Testosterone inhibits adipogenic differentiation in 3T3-L1 cells: nuclear translocation of androgen receptor complex with beta-catenin and T-cell factor 4 may bypass canonical wnt signaling to down-regulate adipogenic transcription factors. Endocrinology 147:141-154.

Sirianni R, Chimento A, Malivindi R, Mazzitelli I, Ando S, Pezzi V. 2007. Insulin-like growth factor-I, regulating aromatase expression through steroidogenic factor 1, supports estrogen-dependent tumor Leydig cell proliferation. Cancer Res 67:8368-8377.

Sirianni R, Chimento A, De Luca A, Zolea F, Carpino A, Rago V, Maggiolini M, Andò S, Pezzi V. 2009. Inhibition of cyclooxygenase-2 down-regulates aromatase activity and decreases proliferation of leydig tumor cells. J Biol Chem 284:28905-28916.

Socas L, Zumbado M, Pérez-Luzardo O, Ramos A, Pérez C, Hernández JR, Boada LD. 2005. Hepatocellular adenomas associated with anabolic androgenic steroid abuse in bodybuilders: a report of two cases and a review of the literature. Brit J Sports Med 39:e27.

Song RXD, Fan P, Yue W, Chen Y, Santen RJ. 2006. Role of receptor complexes in the extranuclear actions of estrogen receptor alpha in breast cancer. Endocr-Relat Cancer 13:S3-13.

Sylvester PW. 2011. Optimization of the tetrazolium dye (MTT) colorimetric assay for cellular growth and viability. Methods Mol Biol 716:157-68.

Tora L, Mullick A, Metger D, Ponglikitmongkol M, Park I, Chambon P. 1989. The cloned human oestrogen receptor contains a mutation which alters its hormone binding properties. EMBO J 8:1981-1986.

Wakabayashi T, Onda H, Tada T, Iijima M, Itoh Y. 1984 High incidence of peliosis hepatis in autopsy cases of aplastic anemia with special reference to anabolic steroid therapy. Acta Pathol Jap 34:1079-1086.

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. Endocrinol 144:5058-5064.

Zhou J, Bondy C. 1993. Anatomy of the insulin-like growth factor system in the human testis. Fertil Steril 60:897-904.

FIGURE LEGENDS

Figure 1: Effects of Nandrolone and Stanozolol on Aromatase expression and estradiol production in R2C cells. (A and B) R2C cells were left untreated (basal) or treated for 24h with nandrolone (nandro) (A) or stanozolol (stano) (B) at the indicated concentrations. Cell proliferation was assessed using the MTT method as indicated in the Materials and Methods section. Final results represent mean ± S.D. of three independent experiments each performed in triplicate. (*, P<0.001 compared with basal). (C and D) R2C cells were left untreated (basal) or treated for 24h with nandrolone (nandro) (C) or stanozolol (stano) (D) at the indicated concentrations. Western blot analysis for aromatase was performed on 50 μg of total proteins. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control. Graphs represent means of Aromatase optical densities normalized to GAPDH content of the same sample. (*, P<0.001 compared with basal). (E) Cells were treated with nandrolone (nandro) (1 μM) or stanozolol (stano) (1 μM) for 48h. E2 content in cell culture medium, measured by RIA, was normalized to the well protein content. Values represent means ± S.D. of three different experiments, each performed in triplicate (*, P<0.001 compared with basal). (F) R2C cells were left untreated (basal) or treated for 48h with nandrolone, (nandro) (1 μM), stanozolol (stano) (1 μM) and ICI182,780 (ICI) (10 μM) alone or in combination. Proliferation was assessed using the MTT method as indicated in the Materials and Methods section. Final results represent mean ± S.D. of three independent experiments. (*, P<0.001 compared with basal; **, P<0.001 compared with nandro; °, P<0.005 compared with stano).

Figure 2: Additive effects of nandrolone or stanozolol and IGF-I on cell proliferation and aromatase expression and activity. (A) R2C cells were left untreated (basal) or treated for 48h with IGF-I (100 ng/ml), nandrolone (nandro) (1 μM), stanozolol (stano) (1 μM) and ICI182,780 (ICI) (10 μM) used alone or in combination. Cell proliferation was assessed using the MTT method as indicated in the Materials and Methods section. Final results represent mean ± S.D. of three independent experiments each performed in triplicate. (*, P<0.001 compared with basal; °, P<0.001 compared with IGF-I; **, P<0.005 compared with IGF-I+nandrolone; $^{\#}$ P<0.001 compared with IGF-I+stano). (B and C) R2C cells were left untreated (basal) or treated for 24h with IGF-I (100 ng/ml) used alone or in combination with nandro (1 μM) (B) or stano (1 μM) (C). Western blot analysis for aromatase was performed on 50 μg of total proteins. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control. Aromatase optical densities were normalized to GAPDH content of the same sample. Graphs represent means

of values from three blots, where basal values were assumed as 100. (*, P<0.001 compared with basal). (D) R2C cells were left untreated (basal) or treated for 48h with nandro (1 μM) or stano (1 μM) and IGF-I (100 ng/ml) alone or in combination. Aromatase activity was assessed using the modified tritiated water method. Result obtained were measured in pmoles of released $[^3H]H_20$, normalized to the well protein content (pmol/mg protein/h) and expressed as fold over basal. Values represent the mean ± S.D. of three independent experiments each performed in triplicate (*, P<0.001 compared with basal; **, P<0.001 compared with IGF-I).

Figure 3: Effects of IGF-I pathway inhibitors on nandrolone and stanozolol induced cell proliferation and aromatase expression. (A-D) R2C cells were left untreated or treated in serum-free medium for 48h (A) or 24h (B-D) with nandrolone (nandro) (1 μM) or stanozolol (stano) (1 μM) alone or in combination with AG1024 (10 μM) (AG), LY294002 (10 μM) (LY), PD98059 (10 μM) (PD) and GF109203X (GFX) (10 μM). (A) Cell proliferation was assessed using the MTT method as indicated in the Materials and Methods section. Final results represent mean ± S.D. of three independent experiments each performed in triplicate. (*, P<0.001 compared with basal; **, P<0.001 compared with nandrolone; +, P<0.001 compared with stanozolol). (B) Total RNA was extracted from cells untreated (basal) and treated as indicated. RT-PCR was used to analyze mRNA levels of CYP19. L19 was used as housekeeping gene. Negative control (neg) was obtained using water instead of cDNA. Image is representative of three independent experiments with similar results. (C and D) Western blot analysis for aromatase was performed on 50 μg of total proteins. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control. Aromatase optical densities were normalized to GAPDH content of the same sample. Graphs represent means of values from three blots, where basal values were assumed as 100. (*, P<0.001 compared with basal; **, P<0.001 compared with nandrolone; +, P<0.001 compared with stanozolol).

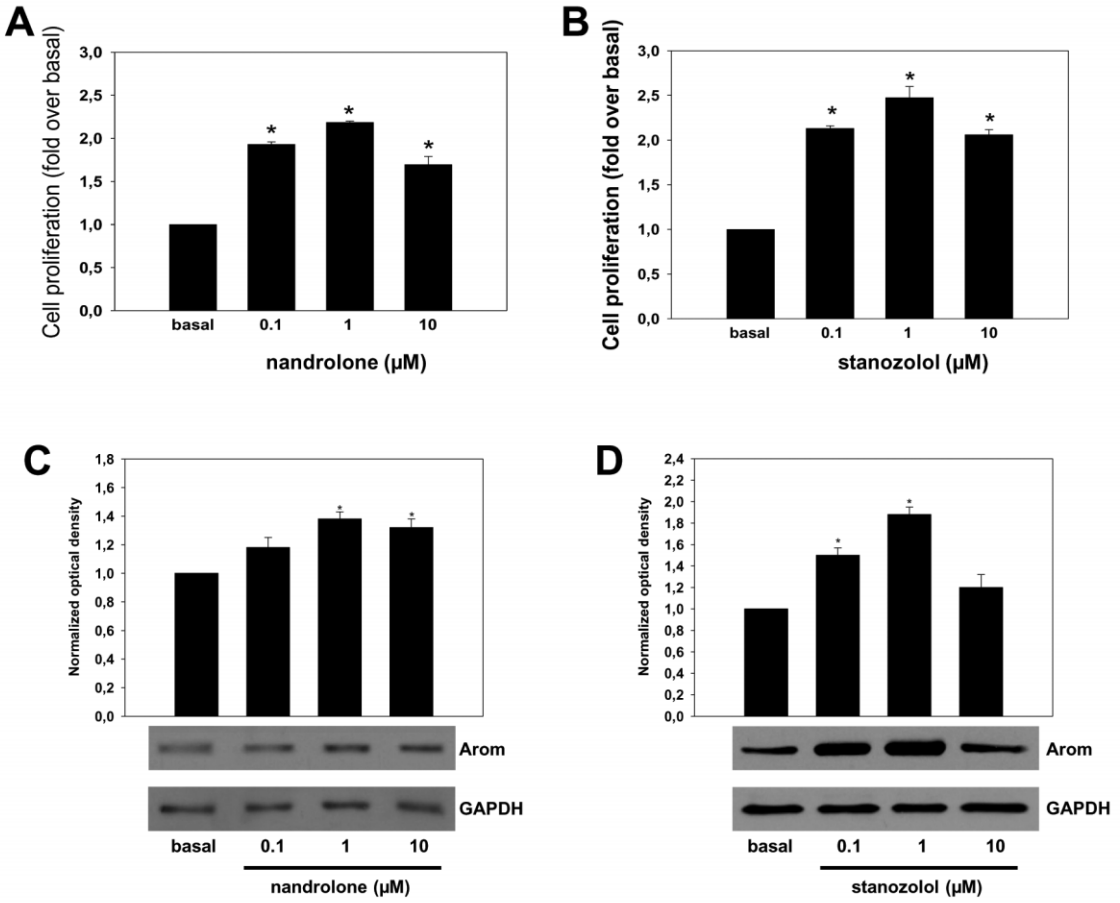
Figure 4: Nandrolone and stanozolol effects on cell proliferation depend on IGF-I production. (A) R2C cells were left untreated (basal) or treated for 48h with nandrolone (nandro) (1 μM) or stanozolol (stano) (1 μM) in 1% DCC. IGF-I levels in culture medium were determined by RIA and IGF-I content was normalized to the cell culture well protein content. Data represent the mean ± SEM of values from three separate cell culture wells expressed as fold over basal. (*) P< 0.01 compared with basal conditions. (B) Cell proliferation was assessed using the MTT method as indicated in the Materials and Methods section. IGF-I antibody (IGF-I Ab) was added to the

medium at 5 μg/ml 24h before being treated for an additional 24h with the indicated concentrations of nandrolone and stanozolol. Columns, mean percent of untreated (basal) cells (100%) from three independent experiments each done in triplicate; bars, S.D. *, $P < 0.01$ compared with basal.

Figure 5: Effects of nandrolone or stanozolol on ER-activated IGF1R signalling. R2C cells were left untreated (basal) or treated for 10 min with nandrolone (nandro) (A) or stanozolol (stano) (B) at the indicated concentrations. (C) R2C cells were left untreated (basal) or treated for 10 min with E2 (100 nM), dihydrotestosterone (DHT) (100 nM), Testosterone (T) (100 nM), nandrolone (1 μM), stanozolol (1 μM) combined with AG1024 (10 μM) (AG), PD98059 (10 μM) (PD) and LY294002 (10 μM) (LY). (D) R2C cells were left untreated (basal) or treated for 10 min with nandrolone (1 μM), stanozolol (1 μM) and ICI182,780 (ICI) (10 μM) used alone or in combination. Western blot analysis was performed on 50 μg of total proteins. Blots are representative of three independent experiments with similar results. GAPDH (A, B, D) or alternatively IGF1R, ERK1/2 and AKT (C) were used as loading control. (E) HEK293 cells were transiently transfected using XETL reporter plasmid and ERα expression vector and treated with the indicated doses of nandrolone or stanozolol. Data were normalized to the coexpressed β-galactosidase expression vector and expressed as RLU. Results represent the mean + S.D. of data from three independent experiments, each performed in triplicate. (*, $P<0.001$ compared with basal).

Figure 6: Additive effects of nandrolone or stanozolol and IGF-I on cyclin E expression in R2C cells. R2C cells were left untreated (basal) or treated for 24h with nandrolone (nandro) (A) or stanozolol (stano) (B) at the indicated concentrations, nandrolone (nandro) (1 μM) plus IGF-I (100 ng/ml) (C) or stanozolol (stano) (1 μM) plus IGF-I (100 ng/ml) (D). Western blot analysis for cyclin E was performed on 50 μg of total proteins. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control. Cyclin E optical densities were normalized to GAPDH content of the same sample. Graphs represent means of values from three blots, where basal values were assumed as 100. (*, $P<0.001$ compared with basal; +, $P<0.001$ compared with IGF-I).

Figure 1



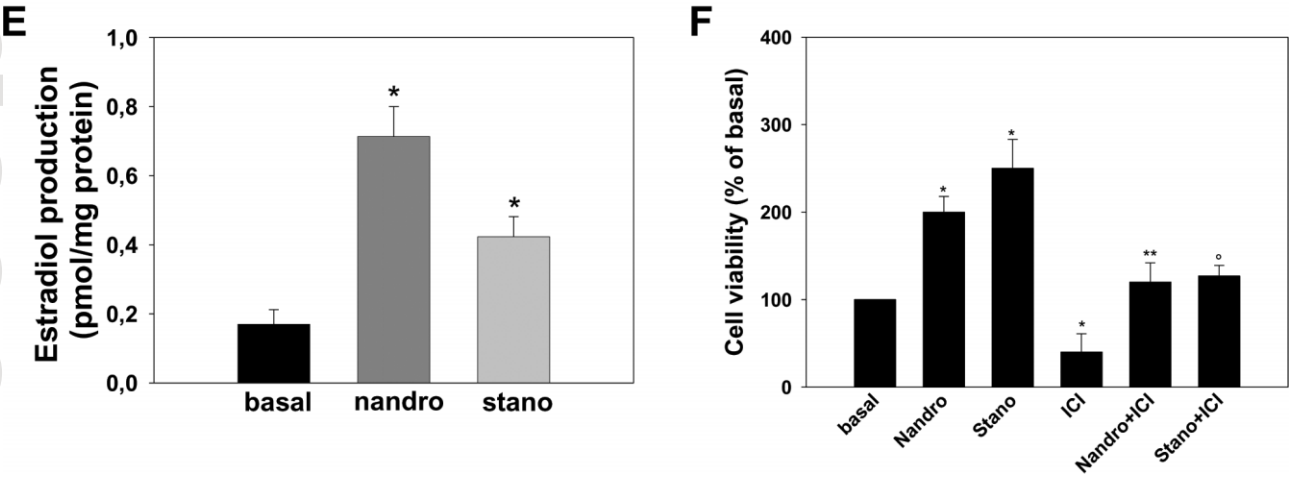
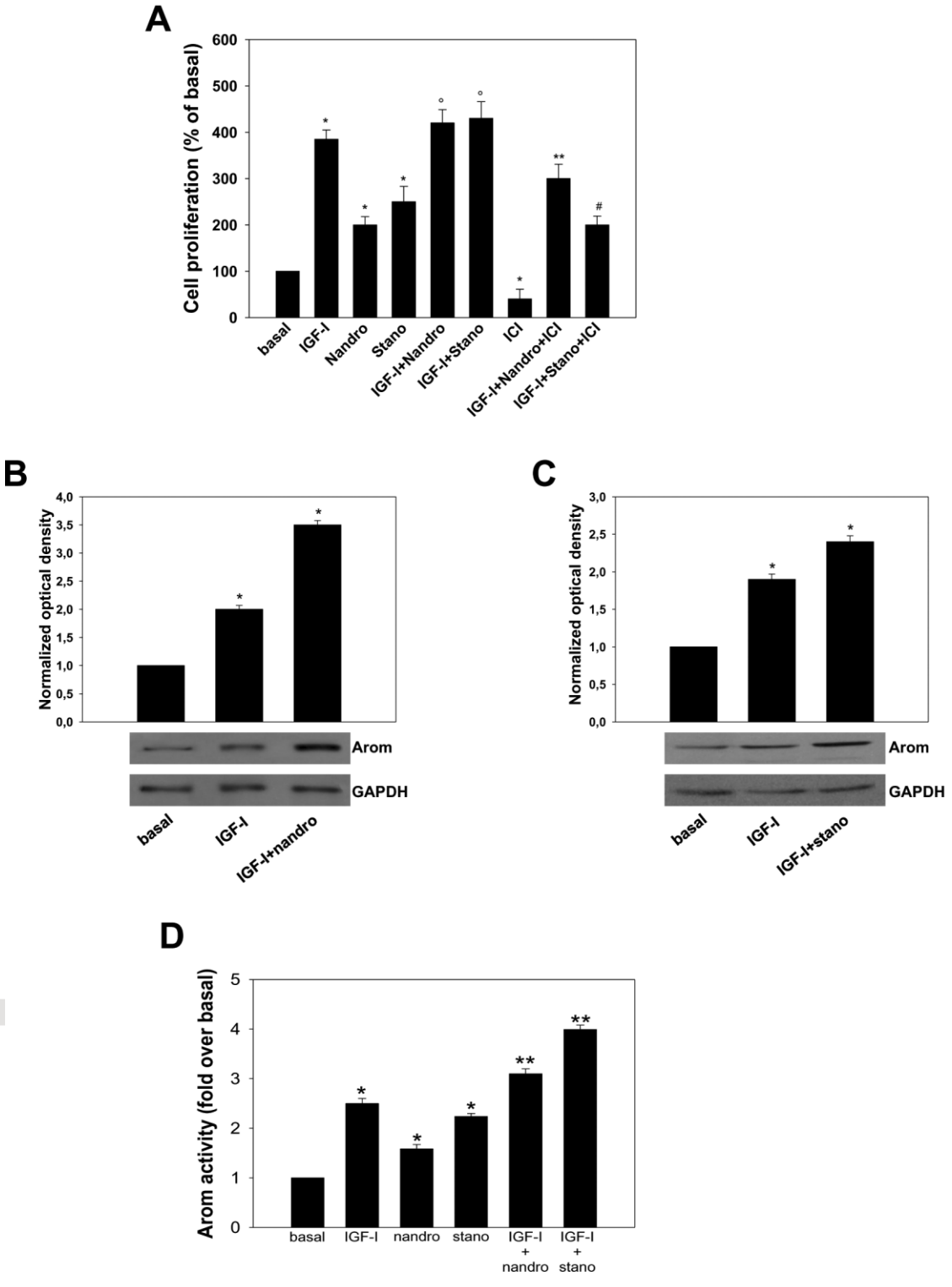


Figure 2



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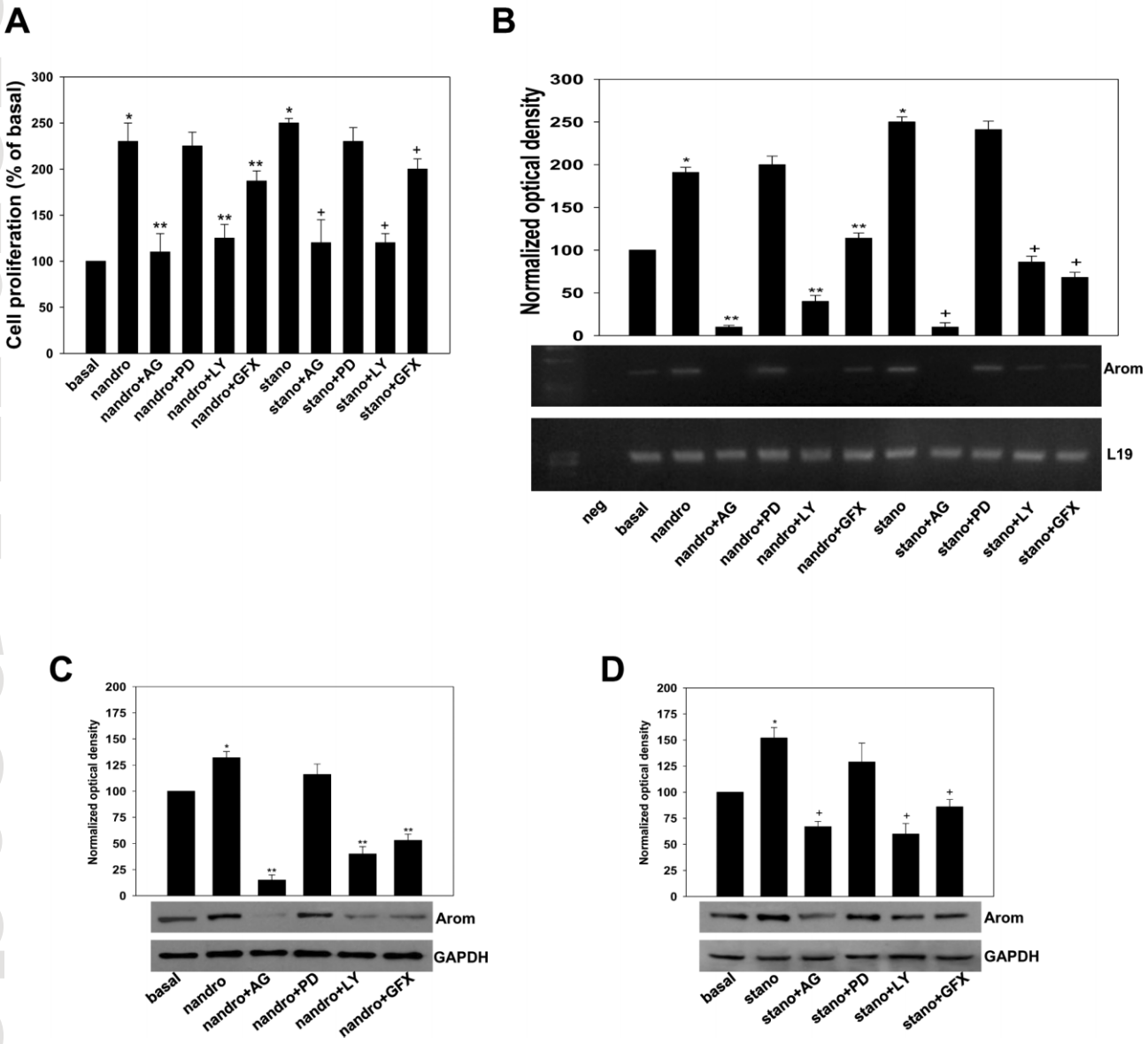

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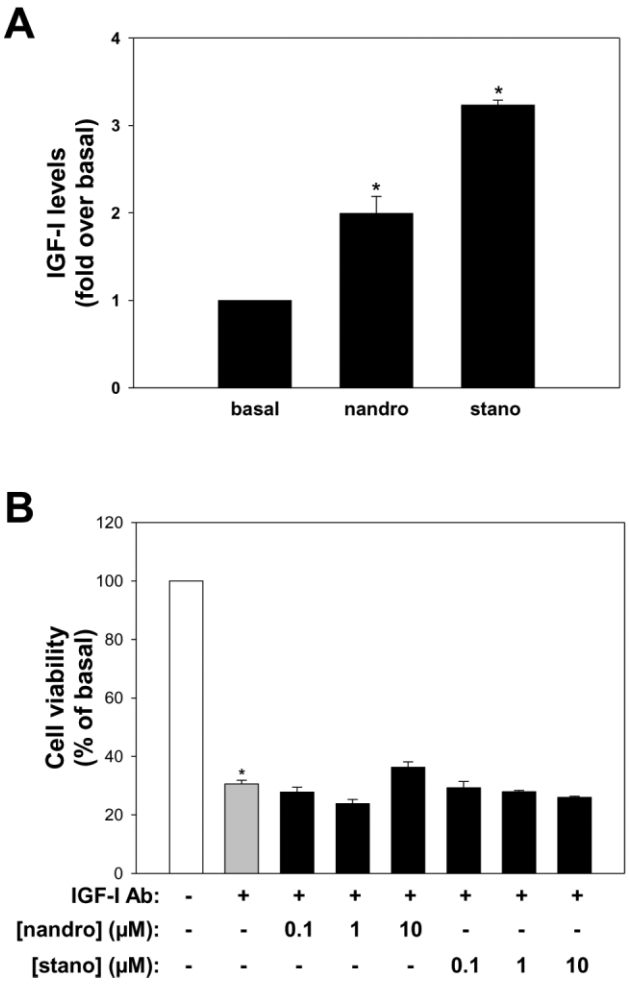

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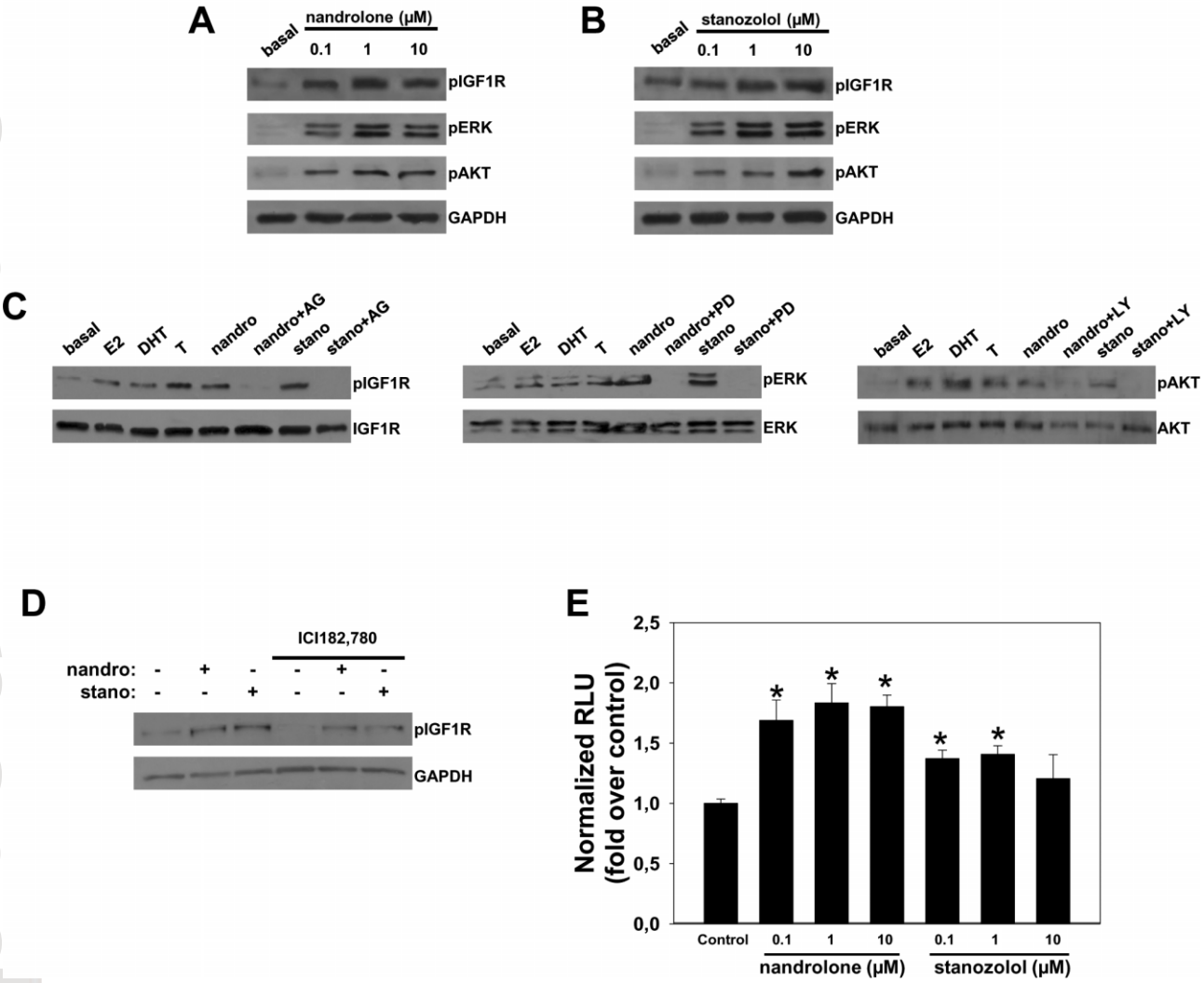

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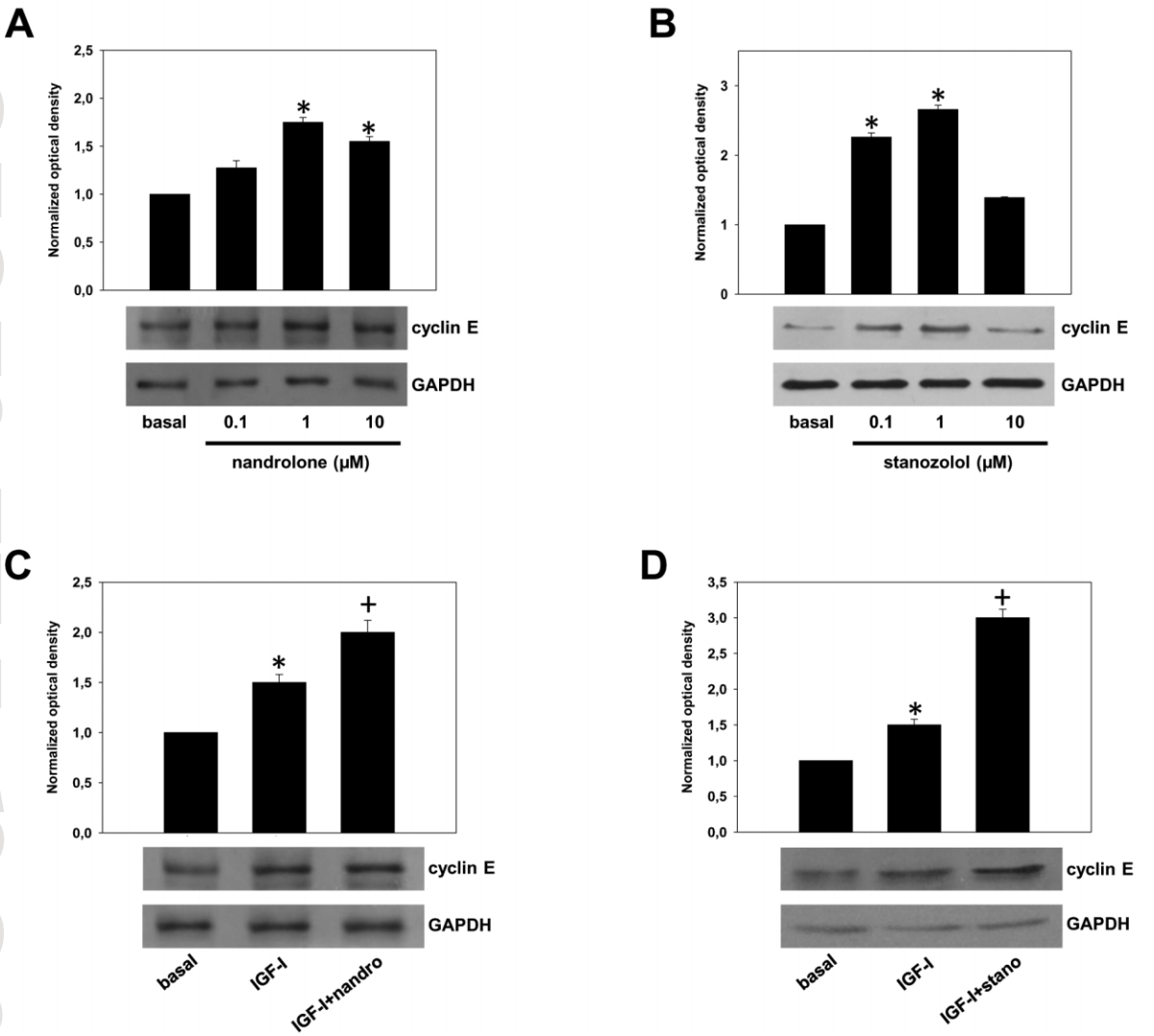

A cross-talk between estrogen and IGF-II signaling controls adrenocortical tumor cell proliferation

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Running title: Role of ERα in ACC proliferation

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Abbreviations: ACC, adrenocortical carcinoma; IGF-II, Insulin-like growth factor-II; IGF1R, IGF receptor; ERα, estrogen receptor α; E2, 17β-estradiol; SF-1, steroidogenic factor-1; ERK1/2, extra-cellular signal-regulated kinase 1/2.

ABSTRACT

Adrenocortical carcinoma (ACC) is characterized by an increased production of insulin-like growth factor-II (IGF-II) and by estrogen receptor (ER) α up-regulation. The objective of this study was to define the interactions between IGF-II and estrogen signaling in ACC, investigating the role played by ERα in IGF-II-dependent tumor proliferation, in order to give new indications for a better therapy of ACC. The role of ERα in ACCs is clarified using H295R cells that we show to display features in common with human ACC tissues: IGF-II-dependent activation of downstream effector pathways and over-expression of estrogen-related genes including ERα and aromatase, the enzyme required for estrogen production. We show that IGF-II controls expression of steroidogenic factor-1 (SF-1), a nuclear receptor that in turn increases aromatase transcription. 17β-Estradiol (E2) bound to ERα up-regulates IGF1R expression increasing pCREB binding to IGF1R promoter. Furthermore, ERα is involved in E2- and IGF-II-dependent cyclin D1 expression, a gene controlling cell cycle progression overexpressed in ACC. Silencing ERα significantly blocked the ability of E2 and IGF-II to induce cell proliferation more effectively than an anti-IGF1R monoclonal antibody, currently used in clinical trials. These findings provide rationale for targeting ERα to control the proliferation of adrenocortical carcinoma.

Keywords: ACC, IGF-II, estrogen, ERα, aromatase

INTRODUCTION

The cause of adrenal cancer remains elusive, but studies in the past 10 years suggest genetic mutations in the adrenal gland leading to the initiations of a malignant tumor (1, 2). The most consistent and dominant genetic change in adrenocortical carcinoma (ACC) is the perturbation of the insulin-like growth factor II (IGF-II) locus (11p15) that is imprinted. IGF-II is over-expressed in 90% of ACCs together with IGF1R. The direct involvement of IGF-II/IGF1R system in adrenocortical tumor cell proliferation has also been shown in vitro using adrenal cancer cell line H295R (3). It has been shown in several tissues that upon ligand binding, the intrinsic tyrosine kinase of the IGF1R caused the activation of phosphatidylinositol 3-kinase (PI3K)/AKT (4) and Raf-1/MEK/ERK pathways regulating positively cellular proliferation. In addition, receptors for growth factors like IGF-II are able to activate also protein kinase C (PKC).

Transgenic mice over-expressing IGF-II postnatally were generated and were demonstrated to have adrenocortical hyperplasia, although frank malignancy was not observed (5). This observation suggests that IGF-II is important for the abnormal proliferation of adrenal cells, but that additional steps are required for transformation to neoplasia. In addition, anti-IGF1R monoclonal antibody figitimumab has been used in phase-I clinical trial for the treatment of refractory adrenocortical carcinoma, however no objective responses were seen in the refractory ACC patients (6).

Epidemiological and experimental studies suggest that estrogens could also be involved in the genesis of ACC. A type of ACC is named feminizing because secrete estrogens leading to gynecomastia and testicular atrophy in men. Moreover, usually ACCs are more frequent in women than in men, especially in those exposed to estro-progestin (7, 8). CYP19 gene is the enzyme responsible for estrogen synthesis using androgens as substrate (9). CYP19 is not usually considered a member of the adrenocortical cytochrome P450 family, however, recent case reports have demonstrated local aromatase cytochrome P450 (CYP19) mRNA and aromatase activity in feminizing human adrenal tumors (10, 11).

Aromatase has been shown in H295R adrenocortical cancer cell line by determination of mRNA (12) as well as enzyme activity (13, 14). We have shown that H295R cells exhibit estrogen-sensitive proliferation which can be inhibited by exposure to antiestrogens ICI182,780 and hydroxytamoxifen (OHT) or to aromatase inhibitor letrozole (14). Estrogens produced by aromatase act by binding nuclear receptor family members ERα and β. We have demonstrated that ACCs are characterized by ERα up-regulation (11), which seems to mediate estrogen-dependent proliferative effects (14). Activated ERs bind to responsive elements (ERE) present on certain gene promoters leading to a change in gene transcription. In particular, ERα can bind AP-1 and Sp1 sites present within the promoter region of cyclin D1 gene (CCND1) increasing its transcription (15). In addition to nuclear responses, estrogens activate rapid cellular responses known as non-genomic steroid signals (16). Through these signals estrogens are able to activate pathways controlling transcription factors.

A number of studies have shown that estrogens and the IGF system may functionally interact and potentiate proliferating effects of the single agents in several tumor tissues, including breast, ovary and endometrial cancer (17-19). Estrogens increase expression of IGF-II (20), IGF-I binding and IGF1R mRNA expression in breast cancer MCF-7 cells (21), whereas the antiestrogen ICI182,780 decreases IGF1R mRNA levels (22) and anti-estrogens, like tamoxifen, can inhibit IGF-mediated growth (23, 24). However, if similar interactions occur in ACC is unknown.

Aim of this study was to investigate the interactions between estrogen and IGF system in ACC and define their role in adrenal tumor cell proliferation. In particular, we wanted to investigate if *in situ* estrogen production could i) be influenced by IGFII-dependent pathways involved in the regulation of aromatase expression; ii) induce IGF1R expression, activating an autocrine loop. Finally, we wanted to compare the ability of IGF1R monoclonal antibody and ERα siRNA in controlling E2- and IGFII-dependent H295R cell proliferation, in order to give new indications for a better therapy for ACC.

MATERIALS AND METHODS

Cell culture and tissues

H295R cells were obtained from Dr W.E. Rainey (Medical College of Georgia, Augusta, GA, USA) (25). Cells were cultured as previously shown (14). Cell monolayers were subcultured onto 100 mm dishes for ChIP assay (8 x 10^6 cells/plate), 60 mm dishes for protein and RNA extraction (4 x 10^6 cells/plate) and 24 well culture dishes for proliferation experiments (2 x 10^5 cells/well) and grown for 2 days. Prior to experiments, cells were starved overnight in DMEM/F-12 medium containing antibiotics. Cells were treated with 17β-estradiol (100 nM) (Sigma, St. Louis, Missouri, USA), 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT) (1 μM) (Tocris Bioscience, Ellisville, Missouri, USA), IGF-II (100 ng/ml) (Sigma), AG1024 (AG) (10 μM) (Sigma), PD98059 (PD) (10 μM) (Calbiochem, Merck KGaA, Darmstadt, Germany), LY294002 (LY) (10 μM) (Calbiochem), GF109203X (GFX) (5 μM) (Calbiochem) and IGF1R blocking antibody αIR3 (1 mg/ml) (Abcam, Cambridge, UK).

Fresh-frozen samples of adrenocortical tumors removed at surgery and normal adrenal cortex macroscopically dissected from adrenal glands of kidney donors were collected at the hospital-based Divisions of the University of Padua and Ancona (Italy). Tissue samples were obtained with the approval of local ethics committees and consent from patients, in accordance with the Declaration of Helsinki guidelines as revised in 1983. Diagnosis of malignancy was performed according to the histopathologic criteria proposed by Weiss et al. (26) and the modification proposed by Aubert et al. (27).

RNA extraction, reverse transcription and PCR

Cells were treated for 24 h. Total RNA was extracted with the TRizol RNA isolation system (Invitrogen Carlsbad, CA, USA). One microgram of RNA from each sample was used for RT-PCR with ImProm-II reverse transcriptase system kit (Promega Madison, WI, USA). qPCR was performed using SYBR Green Universal PCR Master Mix (Bio-Rad, Hercules, CA, USA) using

IGF1R specific primers: Forward 5'-AAGGCTGTGACCCTCACCAT-3'; Reverse 5'-CGATGCTGAAAGAACGTCCAA-3' (Genbank acc. N. NM_000875). Each sample was normalized to its 18S rRNA content as previously shown (28). Human 18S rRNA primers were purchased from Applied Biosystems (Foster City, CA, USA).

Western blot analysis

Phosphorylation levels were determined 10 min after treatment, when inhibitors were used they were added 30 min before stimulus. Changes in expression levels were analysed 24 h after treatment. Fifty µg of proteins was subjected to western blot (29). Blots were incubated overnight at 4°C with specific antibodies: a) anti-pIGF1R antibody (Y1135) (DA7A8) (1:500; Cell Signaling Technology, Beverly, MA, USA), b) anti-IGF1Rβ antibody (C-20) (1:800; Santa Cruz Biotechnology, Santa Cruz, CA, USA), c) anti-pERK1/2 antibody (T202/Y204) (1:500; Cell Signaling Technology), d) anti-ERK1/2 antibody (1:1000; Cell Signaling Technology), e) anti-pAKT1/2/3 (Ser473)-R (1:500; Santa Cruz Biotechnology), f) anti-AKT1/2/3 (H-136) (1:500; Santa Cruz Biotechnology), g) anti-human P450 aromatase antibody (1:200; Serotec, Oxford, UK), h) anti-SF-1 (1:10,000; provided by Prof. Ken-ichirou Morohashi National Institute for Basic Biology, Okazaki, Japan), i) anti-pCREB antibodies [Ser133] (1:1,000; Upstate Biotechnology, Temecula, CA, USA), j) anti-CCND1antibody (3H2043) (1:1,000; Santa Cruz Biotechnology), k) anti-pERα (S118) (16J4) (1:500; Cell Signaling Technology), l) anti-pERα (S167) (D1A3) (1:500; Cell Signaling Technology), m) anti-ERα (F-10) antibody (1:1,000; Santa Cruz Biotechnology). 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). To assure equal loading of proteins membranes were stripped and incubated overnight with GAPDH antibody (GAPDH (FL-335) 1:2000; Santa Cruz Biotechnology).

Assessment of cell proliferation

3-[4,5-Dimethylthiaoly]-2,5-diphenyltetrazolium bromide (MTT) assay was conducted to detect cell proliferation. Cells were treated for 48 h in DMEM F-12 medium containing only 2% Pen/Strep. When IGF1R monoclonal antibody was used it was added 12 h before treatment. 48 h after treatment fresh MTT (Sigma) re-suspended in PBS was added to each well (final concentration 0.33 mg/ml). After 30 min incubation, cells were lysed with 1 ml of DMSO (Sigma). Each experiment was performed in triplicates and the optical density was measured at 570 nm in a spectrophotometer.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation was performed as previously shown (28). Extracted DNA was resuspended in 20 µl of Tris-EDTA buffer. A 5 µl volume of each sample and input were used for real time PCR using primers for the CYP19 promoter II: forward, 5′-AACAGGAGCTATAGATGAAC-3′; reverse, 5′-CAGAGATCCAGACTCGCAATG-3′; primers for the CRE site in the IGF1R promoter: forward, 5′-CTCGAGAGAGGCGGGAGAGC-3′; reverse, 5′-GGAGCGGGGCCGAGGGTCTG-3′; primers for the AP-1 site in the CCND1 promoter: forward, 5′-GAGGGGACTAATATTTCCAGCAA-3′; reverse, 5′-TAAAGGGATTTCAGCTTAGCA-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) 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 for the real time experiments, using input Ct values instead of the 18S, calibrator was basal sample.

RNA interference

The ERα siRNA and non targeting siRNA were purchased from Ambion (Applied Biosystems, Austin, TX, USA). Cells were plated into 60 mm dishes at $4x10^6$ cells, for protein extraction, and into 24-well paltes at $2x10^6$ cells for proliferation assay and used for transfection 48 h later. siRNAs were transfected to a final concentration of 50 nM using the Lipofectamine 2000 and was used according to the manufacturer's recommendations (Invitrogen). ERα-specific knockdown was checked by western analysis of proteins extracted from cells transfected for 48 h and then treated for 24 h. Proliferation was evaluated for cells transfected for 24 h and then treated for 48 h.

Data analysis and statistical methods.

All experiments were conducted at least three times and the results were from representative experiments. Data were expressed as mean values ± standard deviation (SD), and the statistical significance between control (basal) and treated samples was analysed with SPSS10.0 statistical software. The unpaired Student's *t* -test was used to compare two groups. $P < 0.05$ was considered statistically significant.

RESULTS

IGF-II signaling is active in human ACCs tissues and in H295R cells and is involved in cell proliferation.

Considering IGFIR up-regulation in human ACCs (2), we investigated expression of genes related to IGF-II signaling in five different human ACCs samples. Western analysis revealed high expression levels of IGFIR and of the phosphorylated form of AKT (pAKT), compared to normal adrenal (**Fig. 1A**), while we did not found a marked increase in the phosphorylated levels of ERK1/2 (pERK1/2) (**Fig. 1A**). In order to determine if H295R cell line is a useful experimental model to study human ACC, we investigated in these cells IGF-II pathway and the effect of IGF pathway inhibitors on cell proliferation. Treating cells for increasing times with IGF-II we found a rapid increase in IGF1R, phosphorylation (**Fig. 1B**). Similarly, ERK1/2 and AKT were rapidly activated by IGF-II, with maximum induction observed 30 min after treatment (**Fig. 1B**). Specificity of these activations was confirmed by the use of inhibitors, AG for IGF1R (**Fig. 1C**), PD for ERK1/2 (**Fig. 1D**), LY for PI3K/AKT (**Fig. 1E**). AG blocked the activation produced by IGF-II on all kinases (**Fig. 1C-E**). In addition, to confirm that IGF-II/IGF1R pathway influences cell cycle in ACC, we evaluated H295R cell proliferation in response to IGF-II used alone or in combination with AG, PD, LY and GFX, a specific PKC inhibitor (**Fig. 1F**). IGF-II induced cell proliferation by 1.3 fold, while the presence of inhibitors blocked both basal and IGF-II-dependent cell proliferation (**Fig. 1F**). The most effective inhibition was seen with AG that produced a 70% decrease in cell proliferation.

SF-1 and aromatase are highly expressed in human ACCs tissues and regulated by IGF-II/IGF1R pathway.

We have previously shown that ACCs are also characterized by aromatase up-regulation (11). We confirmed these data in our ACC samples and showed a marked increase in the levels of phosphorylated cAMP responsive element binding protein (pCREB) and steroidogenic factor-1

(SF-1) (**Fig. 2A**), two transcription factors regulating aromatase expression through the PII promoter (30). In H295R cells aromatase is also highly expressed (14, 30). Here we found that IGF-II up-regulated aromatase expression in a dose dependent manner and the effects on aromatase were dependent on increased expression of SF-1 while we did not found any change in pCREB levels (**Fig. 2B**). Inhibitors for IGF-II pathway AG, LY and GFX were able to decrease IGF-II-induced aromatase and SF-1 expression levels, while the same inhibitors did not affect pCREB levels (**Fig. 2C**). To confirm the involvement of SF-1 in the control of aromatase expression we performed ChIP analysis that revealed increased binding of SF-1 to the human aromatase PII promoter after IGF-II treatment (**Fig. 2D**), this binding was decreased by AG, LY and GFX but not PD. No changes were observed in pCREB binding levels (**Fig. 2E**).

Estrogens induce IGF1R expression through pCREB recruitment to IGF1R gene promoter.

Established a role for IGF-II in the control of aromatase expression and consequently on estrogen production, we wanted to evaluate if estrogens, through ERα, control ACC proliferation and IGF1R expression. A 48 h treatment of H295R with E2 and ERα specific agonist PPT caused a significant increase in cell proliferation (**Fig. 3A**). H295R cells were treated with E2 and PPT for 24 h, and IGF1R expression was evaluated at both mRNA and protein levels (**Fig. 3B, C**). Results obtained demonstrated increase in IGF1R up-regulation in response to E2 and PPT (**Fig. 3B, C**). IGF1R mRNA was up-regulated by E2 and PPT by 2 and 2.5-fold, respectively (**Fig. 3B**). In an attempt to define how estrogens could induce IGF1R expression, we evaluated expression, in response to E2 and PPT, of pCREB, a transcription factor controlling IGF1R gene transcription (31), highly expressed in human ACC samples (**Fig. 2A**). We found that in our cell line pCREB was strongly activated by E2 and PPT treatment (**Fig. 3D**) and ChIP experiments clarified that E2 enhanced by 2-fold pCREB binding to IGF1R promoter (**Fig. 3E**).

In addition, since minutes after administration E2 can activate IGF1R signaling in normal and malignant cells of various origins (32), we wanted to determine if this occurred also in ACC. We

treated cells for increasing times with E2 (**Fig. 3G**) and found that IGF1R, ERK1/2 and AKT were rapidly phosphorylated. To demonstrate that the effect was directly dependent on ERα, we also used PPT that was able to reproduce the effects seen with E2 (**Fig. 3G**).

E2 and IGFII increase cyclinD1 expression via ERα.

Demonstrated the role for ERα in the control of IGF1R expression, we investigated if this receptor could have a role in the control of H295R cell proliferation altering expression levels of cyclin D1, a gene that is associated with both IGF/IGF1R transdutional pathway and ERα genomic activities (33). We first demonstrated an increase in cyclin D1 expression in human ACC tissues (**Fig. 4A**). Treatment with IGF-II, E2 and PPT caused an increase in cyclin D1 protein levels (**Fig. 4B**). Stimulation of CCND1 mRNA transcription occurs through the interaction of ERα with several regulatory regions present in the promoter region such as binding motifs for AP-1 and Sp-1 (34). IGF-I is known to activate ERα transcriptional activity in a ligand-independent manner through phosphorylation on serine 118 and 167 (35, 36). To correlate the increase in cyclin D1 expression under IGF-II treatment with ERα expression we assessed whether IGF-II could increase ERα activation in H295R cells. Ten min treatment with IGF-II caused an increase in ERα phosphorylation on both serines (**Fig. 4C**) and high phosphorylation levels were also found in ACC tissues where we confirmed higher ERα expression (**Fig. 4D**). Furthermore, both IGF-II and E2 increased ERα binding to the AP-1 site of CCND1 promoter (**Fig. 4E**).

ERα silencing blocks E2 and IGF-II-dependent H295R cell proliferation

We then wanted to compare the ability of an IGF1R monoclonal antibody and an ERα siRNA in controlling E2- and IGFII-dependent H295R cell proliferation. The presence of IGF1R blocking antibody αIR3 decreased IGF1R protein levels (**Fig. 5A**) and had an effect on cell proliferation. Basal proliferation decreased by 50%, IGF-II induced a 1.4-fold increase in cell proliferation ad αIR3 decreased this effect by 50%. In the presence of E2 the inhibition was only 25% (**Fig. 5B**). A

specific ERα siRNA allowed silencing of gene expression in H295R cells (**Fig. 5C**) and in these conditions we observed a reduction in basal (70%), E2- (77%) and IGF-II-induced (72%) cell proliferation (**Fig. 5D**).

DISCUSSION

The current therapy for adrenocortical carcinoma includes the use of mitotane, a drug with cytotoxic effects controlling steroid secretion, that however shows modest efficacy on metastatic disease (37). In addition, an anti-IGF1R monoclonal antibody has been used in phase-I clinical trial for the treatment of refractory ACC, however no objective responses were seen in the refractory ACC patients (6). The main purpose of this study was to demonstrate the existence of a cross-talk between IGF-II and estrogens involved in the control of ACC proliferation, in order to determine if estrogens can be targeted to control both E2- and IGF-II-dependent ACC proliferation, giving new indications for ACC therapy.

In the first part of our study we investigated if IGF-II signaling regulates estrogen production in ACC. Our data on human ACC samples demonstrated an increase in the expression of proteins associated with IGF pathways, particularly IGF1R and the phosphorylated form of AKT, but not ERK1/2. A similar expression pattern was found in H295R cells in response to IGF-II treatment. Evaluation of proliferative behavior demonstrated that IGF-II increased cell proliferation, that was blocked by inhibitors for IGF1R, PKC and AKT, but not for ERK1/2, that, then, do not seem to be involved in IGF-II/IGF1R-dependent adrenal tumor cell proliferation. Moreover, these data indicate H295R cells as a good model for studies investigating the molecular mechanisms involved in ACC proliferation, since they display features common to the human tumor. In these cells IGF-II increased aromatase expression controlling SF-1 levels and its binding to the PII promoter, the promoter used in H295R cells (30), with a mechanism similar to what we have previously demonstrated in tumor Leydig cells (29). A very recent publication indicated SF-1 staining in 158 of 161 (98%) analyzed ACC, pointing SF-1 as a valuable immunohistochemical marker for this type of tumor (38), our results showing an up-regulation of SF-1 in ACCs are in agreement with this study.

E2 and IGF-I are capable to induce the same genes, including IGF1R, in ER-positive breast cancer cells (39, 40), for this reason the second aim of our study was to evaluate the effects of estrogens on

IGF1R expression in ACC. We showed that treatment of H295R cells with E2 caused an increase in IGF1R mRNA and protein expression. This mechanism was mediated by an increase in CREB phosphorylation and in its binding to IGF1R promoter. This results correlates well with data on ACC, demonstrating increased CREB phosphorylation and IGF1R over-expression, and explain the role played by the increased pCREB levels in ACC. Our data are in agreement with reports indicating the ability of E2 to regulate IGF1R expression in prostate cancer (41). The use of PPT, a specific ERα agonist, confirms the role for this estrogen receptor isoform in mediating estrogen effects on IGF1R. Potentially, E2 controls CREB phosphorylation through a classical mechanism involving protein kinase A (PKA) activation, since we revealed an increase in PKA activity after 1 h stimulation with estradiol (data not shown). E2 was also able to stimulate IGF1R phosphorylation, independently of IGF-II. This was probably due to ERα rapid non-genomic actions (32) directly interacting with the SH2 region of Src, the p85α regulatory subunit of PI3K, Shc and IGF1R (42-44), confirming the ability of E2 to exhibit IGF-II-like activity in ACC. With these preliminary remarks, the last aim of this study was to demonstrate the central role played by ERα in the control of IGF-II-dependent ACC cell proliferation. We demonstrated in the examined ACC samples over-expression of cyclin D1, a gene amplified and/or over-expressed in a substantial proportion of different human tumors (45), known to respond to both estrogens and IGFs (33). Particularly, IGF-I is known to stimulate cyclin D1 transcription and enhance cyclin D1 mRNA stability and protein levels (19, 46, 47), suggesting a central role for this cell cycle regulator in the control of IGF-dependent tumor proliferation. Herein we demonstrated that ERα, activated by E2, in a ligand dependent, and by IGF-II, in a ligand independent manner, controls cyclin D1 expression. Specifically, IGF-II increased ERα phosphorylation at serine 118 and 167, which results in receptor activation (35, 42, 48). Importantly, as previously shown, we found high ERα levels and demonstrated its phosphorylated status in ACCs. These data highlight the existence of an interaction between IGF-II and ERα in human ACC.

In addition, when ERα was silenced, E2 and IGF-II lost their ability to increase cell proliferation. We also evaluated the ability of an anti IGF1R monoclonal antibody (αIR3), whose specificity is absolute (49), to control E2- and IGF-II-dependent proliferation. αIR3 inhibited basal and IGF-II induced cell proliferation, however little effects were seen on E2-dependent proliferation. Our data indicate that IGF-II dependent proliferative effects in ACC require ERα, highly expressed in ACCs, and possibly explain why IGF1R monoclonal antibodies, recently entered phase-I clinical trials for the therapy of ACC, failed to give objective responses in refractory ACC patients (6). The existence of a cross-talk between IGF1R and ER signaling pathways was shown *in vitro* in MCF-7 cells where ESR1 silencing resulted in decreased IGF-I-induced G1-S phase progression and decreased expression of CCND1 and CCNE (19). In addition, it was shown *in vivo* in the uterus of ERα knockout mice, that the loss of ERα resulted in IGF-I inability to induce uterine nuclear proliferative responses (50). These studies and our previous data, indicating the ability of antiestrogens to control H295R cell proliferation (14), support the idea of targeting estrogens to interfere with IGF-II signaling in ACC.

In conclusion results from this study demonstrate the important role played by ERα in mediating the mitogenic activity of E2 and IGF-II in ACC. Both E2 and IGF-II induce many comparable responses in H295R cells including activation of IGF1R/AKT signaling and CCND1 expression. Our last set of experiments clearly demonstrates that targeting ERα is effective in controlling E2 and IGF-II dependent cell proliferation, and reveals a central role for ERα in the mechanisms controlling ACC cell proliferation. These data support the possibility of using anti-estrogens to the purpose of controlling adrenocortical carcinoma cell proliferation.

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REFERENCES

1. Soon, P. S. H., Libe, R., Benn, D. E., Gill, A., Shaw, J., Sywak, M. S., Groussin, L., Bertagna, X., Gicquel, C., Bertherat, J., McDonald, K. L., Sidhu, S. B., and Robinson, B. G. (2008) Loss of Heterozygosity of 17p13, With Possible Involvement of ACADVL and ALOX15B, in the Pathogenesis of Adrenocortical Tumors. *Ann Surg* **247,** 157-164

2. Giordano, T. J., Kuick, R., Else, T., Gauger, P. G., Vinco, M., Bauersfeld, J., Sanders, D., Thomas, D. G., Doherty, G., and Hammer, G. (2009) Molecular Classification and Prognostication of Adrenocortical Tumors by Transcriptome Profiling. *Clinl Cancer Res* **15**, 668-676

3. Logie, A., Boulle, N., Gaston, V., Perin, L., Boudou, P., Le Bouc, Y., and Gicquel, C. (1999) Autocrine role of IGF-II in proliferation of human adrenocortical carcinoma NCI H295R cell line. *J Mol Endocrinol* **23**, 23-32

4. Giorgetti, S., Ballotti, R., Kowalski-Chauvel, A., Tartare, S., and Van Obberghen, E. (1993) The insulin and insulin-like growth factor-I receptor substrate IRS-1 associates with and activates phosphatidylinositol 3-kinase in vitro. *J Biol Chem* **268**, 7358-7364

5. Weber, M. M., Auernhammer, C. J., Kiess, W., and Engelhardt, D. (1997) Insulin-like growth factor receptors in normal and tumorous adult human adrenocortical glands. *Eur J Endocrinol* **136**, 296-303

6. Haluska, P., Worden, F., Olmos, D., Yin, D., Schteingart, D., Batzel, G., Paccagnella, M., de Bono, J., Gualberto, A., and Hammer, G. (2010) Safety, tolerability, and pharmacokinetics of the anti-IGF-1R monoclonal antibody figitumumab in patients with refractory adrenocortical carcinoma. *Cancer Chemother Pharmacol* **65**, 765-773

7. Hsing, A. W., Nam, J. M., Co Chien, H. T., McLaughlin, J. K., and Fraumeni, J. F., Jr. (1996) Risk factors for adrenal cancer: an exploratory study. *Int J Cancer* **65**, 432-436

8. Barzon, L., Sonino, N., Fallo, F., Palu, G., and Boscaro, M. (2003) Prevalence and natural history of adrenal incidentalomas. *Eur J Endocrinol* **149**, 273-285

9. Santen, R. J., Brodie, H., Simpson, E. R., Siiteri, P. K., and Brodie, A. (2009) History of Aromatase: Saga of an Important Biological Mediator and Therapeutic Target. *Endocr Rev* **30**, 343-375

10. Phornphutkul, C., Okubo, T., Wu, K., Harel, Z., Tracy, T. F., Jr., Pinar, H., Chen, S., Gruppuso, P. A., and Goodwin, G. (2001) Aromatase P450 Expression in a Feminizing Adrenal Adenoma Presenting as Isosexual Precocious Puberty. *J Clinl Endocrinol Metab* **86**, 649-652

11. Barzon, L., Masi, G., Pacenti, M., Trevisan, M., Fallo, F., Remo, A., Martignoni, G., Montanaro, D., Pezzi, V., and Palù, G. (2008) Expression of aromatase and estrogen receptors in human adrenocortical tumors. *Virchows Archiv* **452**, 181-191

12. Staels, B., Hum, D. W., and Miller, W. L. (1993) Regulation of steroidogenesis in NCI-H295 cells: a cellular model of the human fetal adrenal. *Mol Endocrinol* 7, 423-433

13. Watanabe, M., and Nakajin, S. (2004) Forskolin up-regulates aromatase (CYP19) activity and gene transcripts in the human adrenocortical carcinoma cell line H295R. *J Endocrinol* **180**, 125-133

14. Montanaro, D., Maggiolini, M., Recchia, A. G., Sirianni, R., Aquila, S., Barzon, L., Fallo, F., Ando, S., and Pezzi, V. (2005) Antiestrogens upregulate estrogen receptor beta expression and inhibit adrenocortical H295R cell proliferation. *J Mol Endocrinol* **35**, 245-256

15. Sabbah, M., Courilleau, D., Mester, J., and Redeuilh, G. (1999) Estrogen induction of the cyclin D1 promoter: Involvement of a cAMP response-like element. *Proc Natl Acad Sci USA* **96**, 11217-11222

16. Losel, R. M., Falkenstein, E. L. I. S., Feuring, M. A. R. T., Schultz, A. R. M. I., Tillmann, H. C., Rossol-Haseroth, K. A. R. I., and Wehling, M. A. R. T. (2003) Nongenomic Steroid Action: Controversies, Questions, and Answers. *Physiol Rev* **83**, 965-1016

17. Wimalasena, J., Meehan, D., Dostal, R., Foster, J. S., Cameron, M., and Smith, M. (1993) Growth factors interact with estradiol and gonadotropins in the regulation of ovarian cancer cell growth and growth factor receptors. *Oncol Res.* **5**, 325-337

18. Hata, H., Hamano, M., Watanabe, J., and Kuramoto, H. (1998) Role of estrogen and estrogen-related growth factor in the mechanism of hormone dependency of endometrial carcinoma cells. *Oncology*. **55**, 35-44

19. Zhang, S., Li, X., Burghardt, R., Smith, R., III, and Safe, S. H. (2005) Role of estrogen receptor (ER) alpha in insulin-like growth factor (IGF)-I-induced responses in MCF-7 breast cancer cells. *J Mol Endocrinol* **35**, 433-447

20. Osborne, C. K., Coronado, E. B., Kitten, L. J., Arteaga, C. I., Fuqua, S. A. W., Ramasharma, K., Marshall, M., and Li, C. H. (1989) Insulin-Like Growth Factor-ll (IGF-II): A Potential Autocrine/Paracrine Growth Factor for Human Breast Cancer Acting via the IGF-I Receptor. *Mol Endocrinol* **3**, 1701-1709

21. Stewart, A. J., Johnson, M. D., May, F. E., and Westley, B. R. (1990) Role of insulin-like growth factors and the type I insulin-like growth factor receptor in the estrogen-stimulated proliferation of human breast cancer cells. *J Biol Chem* **265**, 21172-21178

22. Huynh, H., Nickerson, T., Pollak, M., and Yang, X. (1996) Regulation of insulin-like growth factor I receptor expression by the pure antiestrogen ICI 182780. *Clin Cancer Res* **2**, 2037-2042

23. Wakeling, A. E., Newboult, E., and Peters, S. W. (1989) Effects of antioestrogens on the proliferation of MCF-7 human breast cancer cells. *J Mol Endocrinol* **2**, 225-234

24. Freiss, G., Rochefort, H., and Vignon, F. (1990) Mechanisms of 4-hydroxytamoxifen anti-growth factor activity in breast cancer cells: Alterations of growth factor receptor binding sites and tyrosine kinase activity. *Biochem Biophys Res Commun* **173**, 919-926

25. Rainey, W. E., Bird, I. M., and Mason, J. I. (1994) The NCI-H295 cell line: a pluripotent model for human adrenocortical studies. *Mol Cell Endocrinol* **100**, 45-50

26. Weiss, L. M., Medeiros, L. J., and Vickery, A. L., Jr. (1989) Pathologic features of prognostic significance in adrenocortical carcinoma. *Am J Surg Pathol* **13**, 202-206

27. Aubert, S. b., Wacrenier, A., Leroy, X., Devos, P., Carnaille, B., Proye, C., Wemeau, J. L., Lecomte-Houcke, M., and Leteurtre, E. (2002) Weiss System Revisited: A Clinicopathologic and Immunohistochemical Study of 49 Adrenocortical Tumors. *The Am J Surg Pathol* **26,** 1612-1619

28. Sirianni, R., Nogueira, E., Bassett, M. H., Carr, B. R., Suzuki, T., Pezzi, V., And•, S., and Rainey, W. E. (2010) The AP-1 family member FOS blocks transcriptional activity of the nuclear receptor steroidogenic factor 1. *J Cell Sci* **123**, 3956-3965

29. Sirianni, R., Chimento, A., Malivindi, R., Mazzitelli, I., Ando, S., and Pezzi, V. (2007) Insulin-like growth factor-I, regulating aromatase expression through steroidogenic factor 1, supports estrogen-dependent tumor Leydig cell proliferation. *Cancer Res* **67**, 8368-8377

30. Nicol, M. R., Papacleovoulou, G., Evans, D. B., Penning, T. M., Strachan, M. W. J., Advani, A., Johnson, S. J., Quinton, R., and Mason, J. I. (2009) Estrogen biosynthesis in human H295 adrenocortical carcinoma cells. *Mol Cell Endocrinol* **300**, 115-120

31. Genua, M., Pandini, G., Sisci, D., Castoria, G., Maggiolini, M., Vigneri, R., and Belfiore, A. (2009) Role of Cyclic AMP Response Element-Binding Protein in Insulin-like Growth Factor-I Receptor Up-regulation by Sex Steroids in Prostate Cancer Cells. *Cancer Res* **69**, 7270-7277

32. Levin, E. R. (2005) Integration of the Extranuclear and Nuclear Actions of Estrogen. *Mol Endocrinol* **19**, 1951-1959

33. Cascio, S., Bartella, V., Garofalo, C., Russo, A., Giordano, A., and Surmacz, E. (2007) Insulin-like Growth Factor 1 Differentially Regulates Estrogen Receptor-dependent Transcription at Estrogen Response Element and AP-1 Sites in Breast Cancer Cells. *J Biol Chem* **282**, 3498-3506

34. Castro-Rivera, E., Samudio, I., and Safe, S. (2001) Estrogen Regulation of Cyclin D1 Gene Expression in ZR-75 Breast Cancer Cells Involves Multiple Enhancer Elements. *J Biol Chem* **276**, 30853-30861

35. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon, P. (1995) Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science.* **270**, 1491-1494

36. Lee, A. V., Weng, C. N., Jackson, J. G., and Yee, D. (1997) Activation of estrogen receptor-mediated gene transcription by IGF-I in human breast cancer cells. *J Endocrinol* **152**, 39-47

37. Terzolo, M., Angeli, A., Fassnacht, M., Daffara, F., Tauchmanova, L., Conton, P. A., Rossetto, R., Buci, L., Sperone, P., Grossrubatscher, E., Reimondo, G., Bollito, E., Papotti, M., Saeger, W., Hahner, S., Koschker, A. C., Arvat, E., Ambrosi, B., Loli, P., Lombardi, G., Mannelli, M., Bruzzi, P., Mantero, F., Allolio, B., Dogliotti, L., and Berruti, A. (2007) Adjuvant Mitotane Treatment for Adrenocortical Carcinoma. *N Engl J Med* **356**, 2372-2380

38. Sbiera, S., Schmull, S., Assie, G., Voelker, H. U., Kraus, L., Beyer, M., Ragazzon, B., Beuschlein, F., Willenberg, H. S., Hahner, S., Saeger, W., Bertherat, J., Allolio, B., and

Fassnacht, M. (2010) High Diagnostic and Prognostic Value of Steroidogenic Factor-1 Expression in Adrenal Tumors. *J Clin Endocrinol Metab* **95**, E161-E171

39. Katzenellenbogen, B. S., and Norman, M. J. (1990) Multihormonal regulation of the progesterone receptor in MCF-7 human breast cancer cells: interrelationships among insulin/insulin-like growth factor-I, serum, and estrogen. *Endocrinology* **126**, 891-898

40. Varma, H., and Conrad, S. E. (2002) Antiestrogen ICI 182,780 Decreases Proliferation of Insulin-like Growth Factor I (IGF-I)-treated MCF-7 Cells without Inhibiting IGF-I Signaling. *Cancer Res* **62**, 3985-3991

41. Pandini, G., Genua, M., Frasca, F., Squatrito, S., Vigneri, R., and Belfiore, A. (2007) 17β-Estradiol Up-regulates the Insulin-like Growth Factor Receptor through a Nongenotropic Pathway in Prostate Cancer Cells. *Cancer Res* **67**, 8932-8941

42. Simoncini, T., Hafezi-Moghadam, A., Brazil, D. P., Ley, K., Chin, W. W., and Liao, J. K. (2000) Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* **407**, 538-541

43. Sun, M., Paciga, J. E., Feldman, R. I., Yuan, Z. q., Coppola, D., Lu, Y. Y., Shelley, S. A., Nicosia, S. V., and Cheng, J. Q. (2001) Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, Activated in Breast Cancer, Regulates and Is Induced by Estrogen Receptor à (ERà) via Interaction between ERà and PI3K. *Cancer Res* **61**, 5985-5991

44. Razandi, M., Oh, P., Pedram, A., Schnitzer, J., and Levin, E. R. (2002) ERs Associate with and Regulate the Production of Caveolin: Implications for Signaling and Cellular Actions. *Mol Endocrinol* **16**, 100-115

45. Weinstein, I. B. (1996) Relevance of cyclin D1 and other molecular markers to cancer chemoprevention. *J Cell Biochem Suppl.* **25**, 23-28

46. Dufourny, B., van Teeffelen, H. A., Hamelers, I. H., Sussenbach, J. S., and Steenbergh, P. H. (2000) Stabilization of cyclin D1 mRNA via the phosphatidylinositol 3-kinase pathway in MCF-7 human breast cancer cells. *J Endocrinol* **166**, 329-338

47. Butt, A. J., McNeil, C. M., Musgrove, E. A., and Sutherland, R. L. (2005) Downstream targets of growth factor and oestrogen signalling and endocrine resistance: the potential roles of c-Myc, cyclin D1 and cyclin E. *Endocr Relat Cancer* **12**, S47-S59

48. Bunone, G., Briand, P. A., Miksicek, R. J., and Picard, D. (1996) Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO J.* **15**, 2174-2183

49. Surmacz, E. (2003) Growth factor receptors as therapeutic targets: strategies to inhibit the insulin-like growth factor I receptor. *Oncogene* **22**, 6589-6597

50. Klotz, D. M., Hewitt, S. C., Ciana, P., Raviscioni, M., Lindzey, J. K., Foley, J., Maggi, A., DiAugustine, R. P., and Korach, K. S. (2002) Requirement of Estrogen Receptor à in Insulin-like Growth Factor-1 (IGF-1)-induced Uterine Responses and in Vivo Evidence for IGF-1/Estrogen Receptor Cross-talk. *J Biol Chem* **277**, 8531-8537

FIGURE LEGENDS

Figure 1. IGF-II signaling is active in human ACCs tissues and in H295R cells and is involved in cell proliferation. A, Immunoblot analyses for IGF1R, pAKT and pERK1/2 were performed on 50 μg of total proteins extracted from human normal adrenal tissues (N) and ACCs (C1-C5). B, Western blot analyses of pIGF1R, pERK1/2 and pAKT were performed on 50 μg of total protein extracted from H295R cells untreated or treated for the indicated times with IGF-II (100 ng/ml). GAPDH was used as a loading control. Results are representative of three different experiments. C, Immunoblot analysis for pIGF1R was performed on 50 μg of total protein extracted from H295R cells untreated or treated for 10 min with IGF-II (100 ng/ml) and AG1024 (10 μM) alone or in combination. IGF1R was used as a loading control. Results are representative of three different experiments. D, Immunoblot analysis for pERK1/2 was performed on 50 μg of total protein extracted from H295R cells untreated or treated for 10 min with IGF-II (100 ng/ml) and AG1024 (10 μM) and PD98059 (10 μM) alone or in combination. Total ERK1/2 was used as a loading control. Results are representative of three different experiments. E, Immunoblot analysis for pAKT was performed on 50 μg of total protein extracted from H295R cells untreated or treated for 10 min with IGF-II (100 ng/ml), AG1024 (10 μM) and LY294002 (10 μM) alone or in combination. Total AKT was used as a loading control. Results are representative of three different experiments. F, H295R cells were left untreated or treated for 48 h with AG1024 (10 μM), PD98059 (10 μM), LY294002 (10 μM), GF109203X (5μM), alone or in combination with IGF-II (100 ng/ml). H295R proliferation was evaluated by MTT assay. Results are representative of three independent experiments. Statistically significant differences are indicated (*, $P<0.05$ compared with basal; **, $P<0.01$, compared with IGF-II).

Figure 2. SF-1 and aromatase are highly expressed in human ACCs tissues and regulated by IGF-II/IGF1R pathway. *A*) Immunoblot analyses of aromatase (Arom), SF-1 and pCREB in human normal adrenal tissues [N] and ACCs [C1-C5]. *B*) Immunoblot analyses of aromatase (Arom), SF-1 and pCREB in H295R cells treated with IGF-II at the indicated doses. *C*) Immunoblot analyses of aromatase (Arom), SF-1 and pCREB in H295R cells treated with AG1024 (10 μM) and PD98059 (10 μM) LY294002 (10 μM), GF109203X (5μM) in combination with IGF-II. GAPDH was used as a loading control. *D, E*) H295R cells were treated with AG, PD, LY, GFX, in combination with IGF-II (100 ng/ml). In vivo binding of SF-1 (*D*) or pCREB (*E*) to the aromatase PII promoter was examined using ChIP assay after 24 h treatment. (*P<0.001 compared with basal; **P<0.001 compared with IGF-II).

Figure 3. Estrogens induce IGF1R expression through pCREB recruitment to IGF1R gene promoter. *A*) H295R cells were left untreated (basal) or treated for 48 h with E2 (100 nM) and PPT (1 μM). H295R cell proliferation was evaluated by MTT assay. Results are representative of three independent experiments. *B*) H295R cells were treated with E2 and PPT. Total RNA was extracted, and real-time RT-PCR was used to analyze IGF1R mRNA levels. *C,D*) Western blot analyses of IGF1R (*C*) and pCREB (*D*). E, After chromatin immunoprecipitation, using an anti-pCREB antibody, total DNA was extracted and real time RT-PCR was used to analyze pCREB binding to IGF1R promoter after 24 h treatment. *F, G*) Immunoblot analysis for pIGF1R, pERK1/2 in H295R cells treated for the indicated times with E2 (*F*) and PPT (*G*). (*P<0.01 compared with basal).

Figure 4. E2 and IGF-II increase ERα recruitment to CyclinD1 promoter. *A, D*) Immunoblot analyses for cyclin D1 (*A*), phospho ERα serine 118 (pERα S118), phospho ERα serine 167 (pERα S167) and ERα (*D*) in human normal adrenal tissues [N] and ACCs [C1-C5]. *B*) and *C*) Immunoblot analyses of cyclin D1 (*B*), pERα S118 and pERα S167 (*C*) were performed on 50 μg of total protein extracted from H295R cells untreated (basal) or treated for 24 h with E2 (100 nM), PPT (1 μM) and

IGF-II (100 ng/ml). GAPDH was used as a loading control. *E*) In vivo binding of ERα to the Cyclin D1 promoter after 1 h treatment was examined using ChIP assay. (*P<0.001 compared with basal).

Figure 5. ERα silencing blocks E2 and IGF-II induced H295R cell proliferation. *A, B*) Where indicated H295R cells were incubated over night with αIR3 before being treated for an additional 48 (*A*) or 24 (*B*) h with IGF-II, E2 or vehicle (basal). *C, D*) H295R cells were transfected with ERα siRNA or a non targeting siRNA (control siRNA). Twenty-four (*C*) or 48 (*D*) hours after transfection cells were treated for an additional 48 h (*C*) or 24 h (*D*) with IGF-II, E2 or vehicle (basal). Immunoblot analyses of IGF1R (*A*) and ERα (*C*) were performed on 50 μg of total protein, GAPDH was used as a loading control. Results are representative of three independent experiments. *B, D*) Reduction of IGF-II- and E2-induced H295R cell proliferation by αIR3 (*B*) siRNA and by ERα (*D*) was evaluated by MTT assay. (*, P<0.0001 compared with basal; **, P<0.001 compared with IGF-II; §, P<0.01 compared with E2).

Figure 1

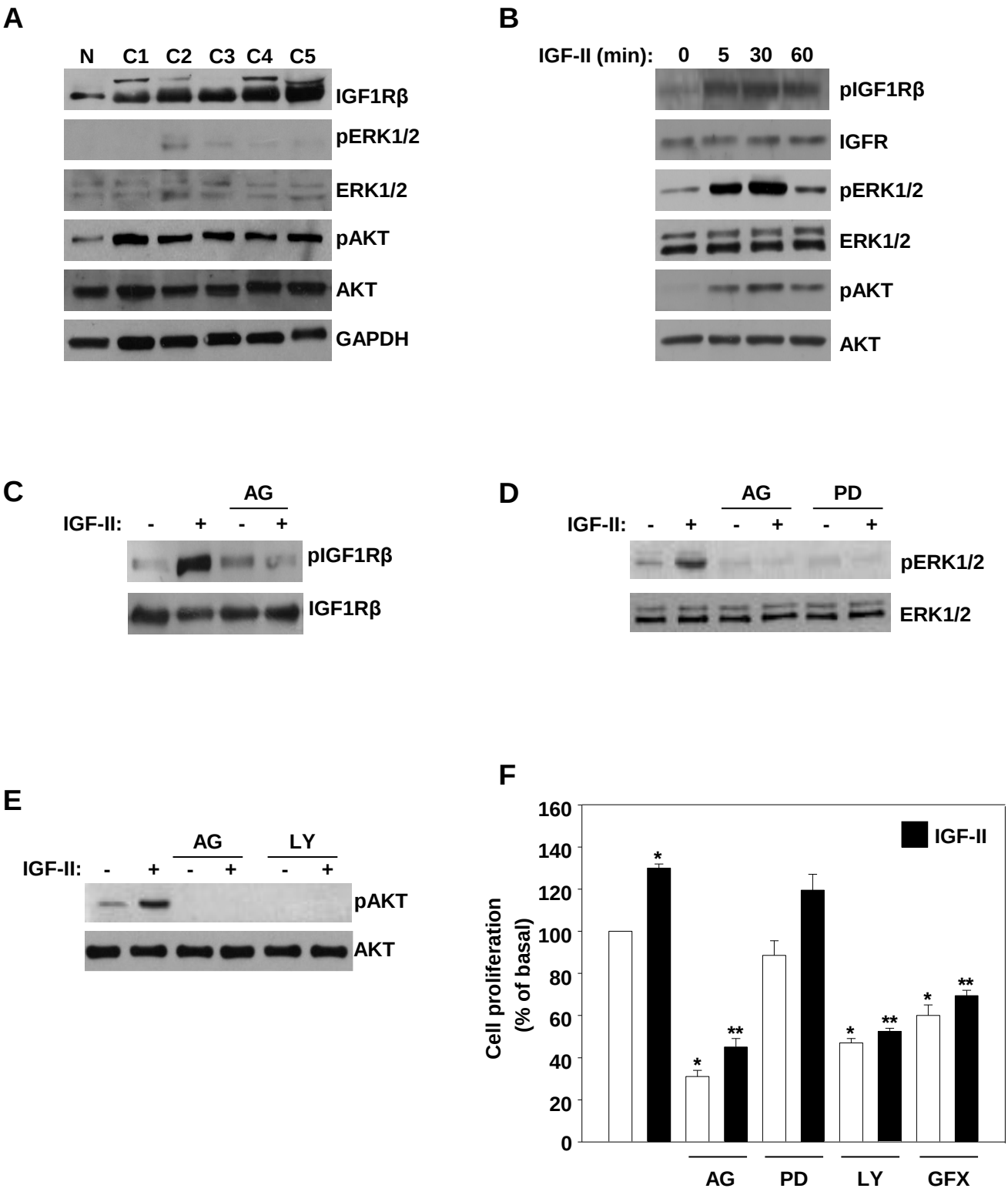

A
N C1 C2 C3 C4 C5
IGF1Rβ
pERK1/2
ERK1/2
pAKT
AKT
GAPDH
B
IGF-II (min): 0 5 30 60
pIGF1Rβ
IGFR
pERK1/2
ERK1/2
pAKT
AKT
C
AG
IGF-II: - + - +
pIGF1Rβ
IGF1Rβ
D
AG PD
IGF-II: - + - + - +
pERK1/2
ERK1/2
E
AG LY
IGF-II: - + - + - +
pAKT
AKT
F
IGF-II
Cell proliferation (% of basal)
160
140
120
100
80
60
40
20
0
AG PD LY GFX

Figure 2

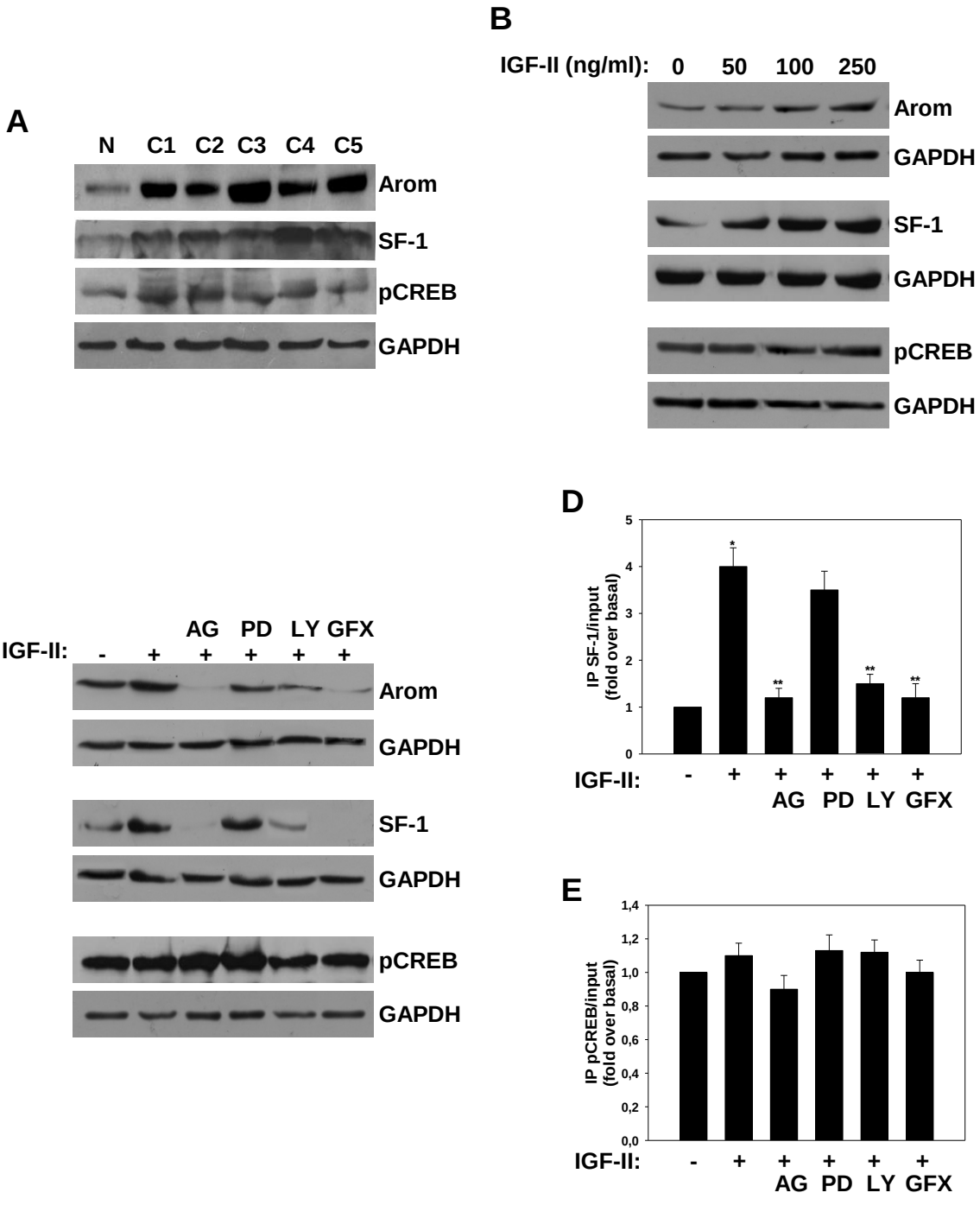

A
N C1 C2 C3 C4 C5
Arom
SF-1
pCREB
GAPDH
B
IGF-II (ng/ml): 0 50 100 250
Arom
GAPDH
SF-1
GAPDH
pCREB
GAPDH
C
IGF-II: - + + + + +
AG PD LY GFX
Arom
GAPDH
SF-1
GAPDH
pCREB
GAPDH
D
IP SF-1/input (fold over basal)
0 1 2 3 4 5
* ** ** **
IGF-II: - + + + + +
AG PD LY GFX
E
IP pCREB/input (fold over basal)
0,0 0,2 0,4 0,6 0,8 1,0 1,2 1,4
IGF-II: - + + + + +
AG PD LY GFX

Figure 3

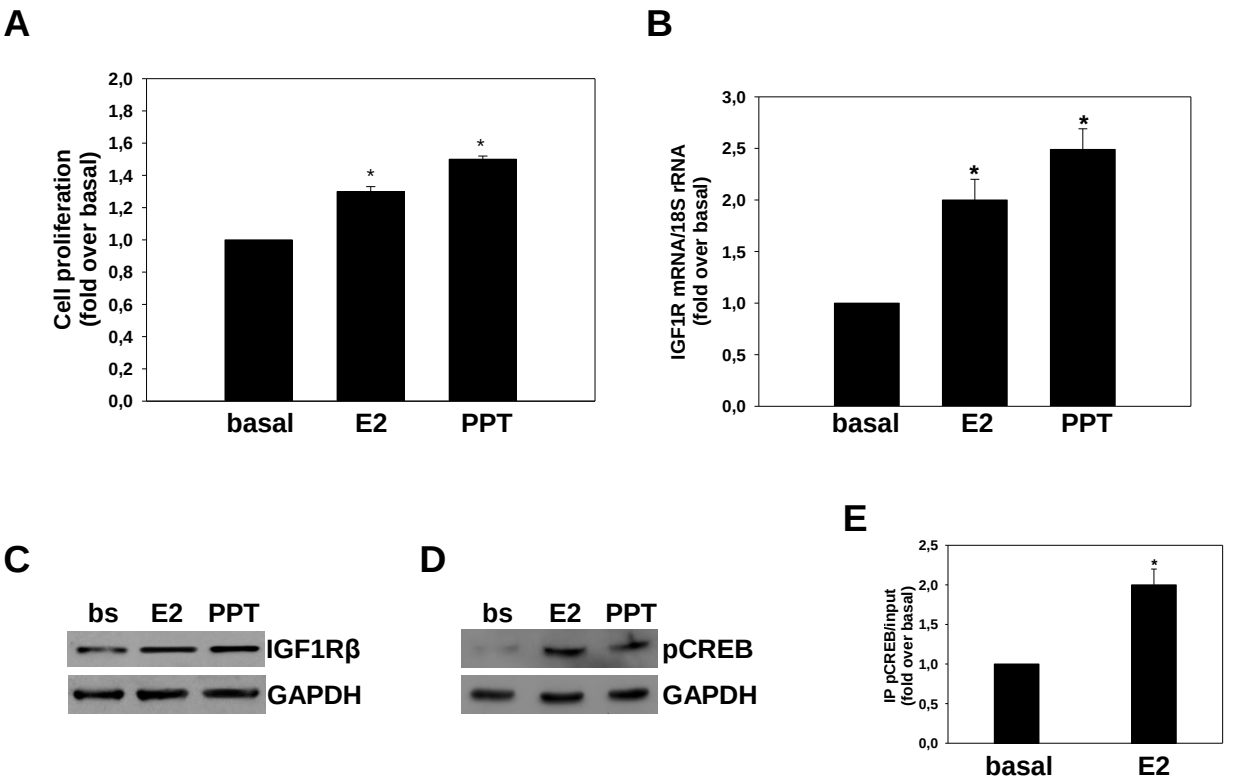
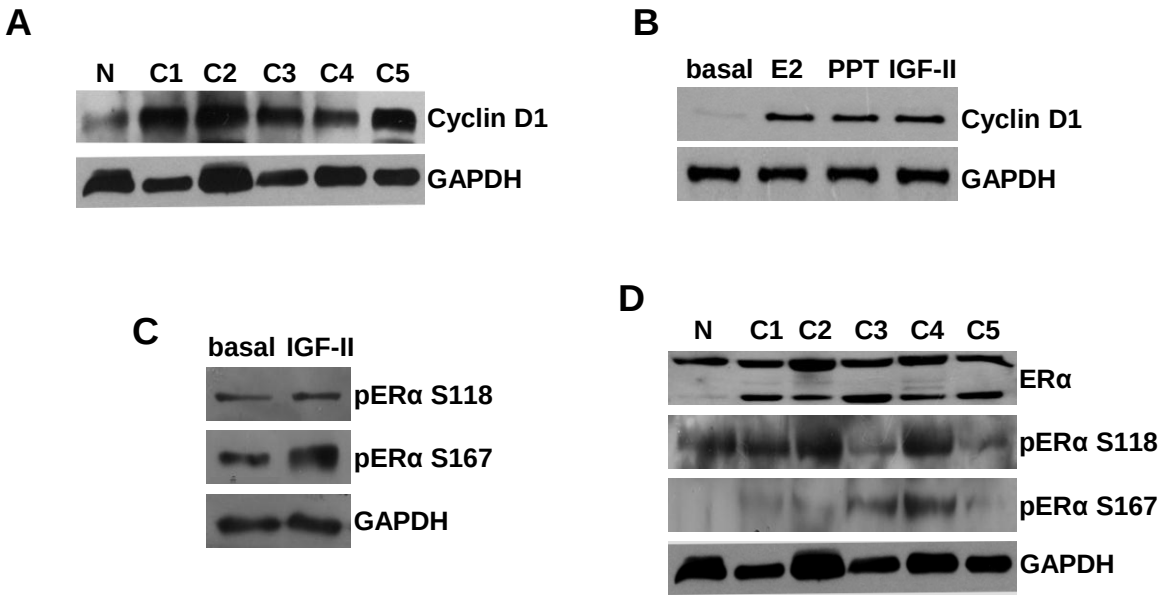

A
Cell proliferation (fold over basal)
2,0
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
*
*
basal
E2
PPT
B
IGF1R mRNA/18S rRNA (fold over basal)
3,0
2,5
2,0
1,5
1,0
0,5
0,0
*
*
basal
E2
PPT
C
bs
E2
PPT
IGF1Rβ
GAPDH
D
bs
E2
PPT
pCREB
GAPDH
E
IP pCREB/input (fold over basal)
2,5
2,0
1,5
1,0
0,5
0,0
*
basal
E2
F
E2 (min):
0
5
10
30
60
pIGF1Rβ
pERK1/2
pAKT
GAPDH
G
PPT (min) :
0
5
10
30
60
pIGF1Rβ
pERK1/2
pAKT
GAPDH

Figure 4


A
N C1 C2 C3 C4 C5
Cyclin D1
GAPDH
B
basal E2 PPT IGF-II
Cyclin D1
GAPDH
C
basal IGF-II
pERα S118
pERα S167
GAPDH
D
N C1 C2 C3 C4 C5
ERα
pERα S118
pERα S167
GAPDH

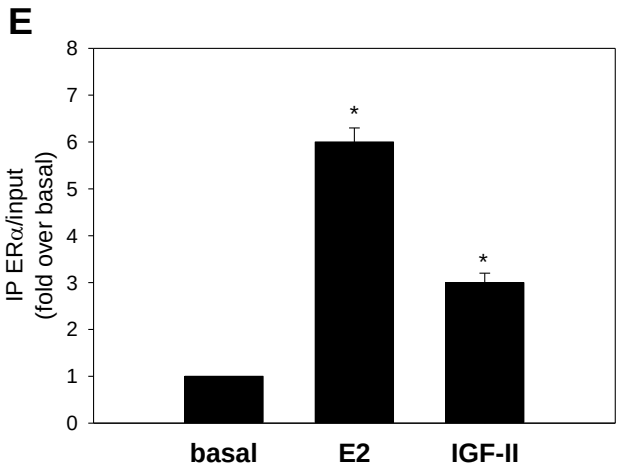
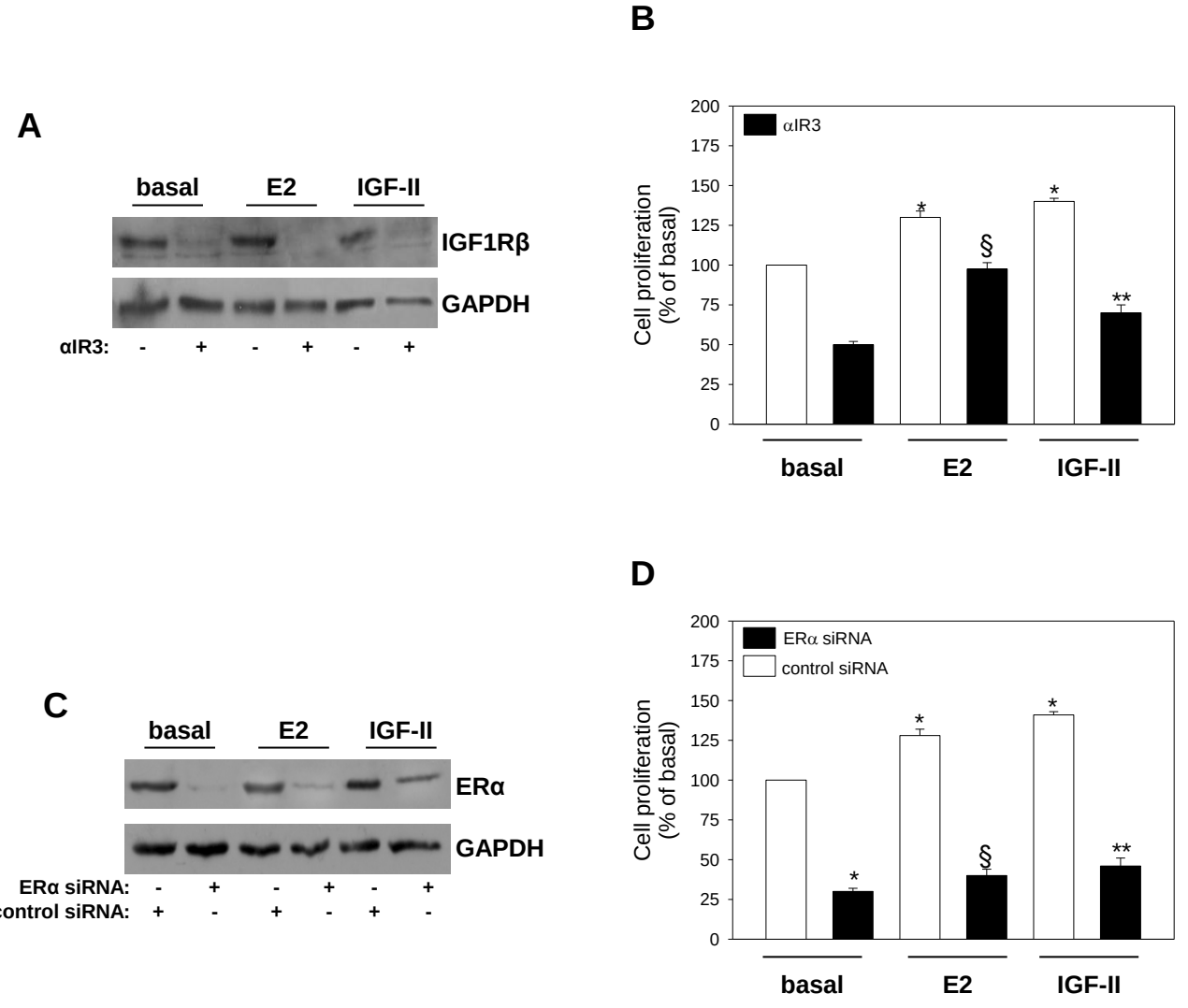

E
IP ERα/input
(fold over basal)
0
1
2
3
4
5
6
7
8
*
*
basal
E2
IGF-II

Figure 5


A
basal
E2
IGF-II
IGF1Rβ
GAPDH
αIR3: - + - + - +
B
αIR3
Cell proliferation (% of basal)
200
175
150
125
100
75
50
25
0
*
§
*
**
basal
E2
IGF-II
C
basal
E2
IGF-II
ERα
GAPDH
ERα siRNA: - + - + - +
control siRNA: + - + - + -
D
ERα siRNA
control siRNA
Cell proliferation (% of basal)
200
175
150
125
100
75
50
25
0
*
*
§
*
**
basal
E2
IGF-II