UNIVERSITY OF ROME

“TOR VERGATA”

FACULTY OF MEDICINE

PHD PROGRAM IN SCIENCES AND BIOTECHNOLOGIES OF REPRODUCTION AND DEVELOPMENT

XXI CYCLE

Effects of estrogens and endocrine disrupters on mouse embryonal germ cells and somatic gonadal cells

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A Francesco per la sua immensa pazienza.

Grazie

Desidero ringraziare il Prof. Massimo De Felici per avermi guidato in tutti questi anni che ho trascorso nel suo laboratorio, per i suoi consigli e soprattutto per avermi insegnato il lavoro del ricercatore.

Desidero ringraziare con grande affetto le persone che con me hanno diviso le gioie ed i dolori della ricerca: Luisa Campagnolo, Donatella Farini e Francesca Klinger (alias le tre ricerchine) per aver sempre dato una risposta alle mie domande "scientifiche" e per avermi dato la risposta più importante nelle situazioni difficili, la loro sensibilità.

Desidero ringraziare le compagne di viaggio: Marianna Tedesco, Lucia Scaldaferri, Elena Ievoli e Ilana Moscatelli con cui ho vissuto dei sereni momenti e le persone che sono passate per il nostro laboratorio alle quali mi lega un ricordo d'affetto, in particolare Enrico Pierantozzi.

Desidero ringraziare inoltre la Prof.ssa Susanna Dolci ed il Prof. Claudio Sette senza il cui aiuto parte dei risultati di questo lavoro non sarebbero esistiti.

Ringrazio infine il Prof. Siracusa, la Prof.ssa Camaioni e la Prof.ssa Salustri per la loro sincera disponibilità.

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Additional Paper Genistein is an Efficient Estrogen in the Whole-Body throughout Mouse Development

Preface

The work presented in this thesis focuses on the effects of estrogens and endocrine disrupters termed xenoestrogens on mouse embryonal germ cells and somatic gonadal cells. The thesis is structured as follows: first, a general introduction to the thesis work; then, present the results obtained in form of one accepted paper for publication, two papers in preparation and one paper published in collaboration with the 3rd Laboratory Biotechnology Civic Hospital of Brescia; finally additional results to the thesis work.

Aims

In the recent years many progresses have been made in order to establish possible causes of anomalies of the reproductive function termed TDS (Testicular Dysgenesis Syndrome) including testicular cancer, cryptorchidism, hypospadias and sterility. Several studies indicate that these anomalies are associated to an increase in the environment of compounds acting like natural hormones named endocrine disrupters (EDs) and in particular to a class of ED that mimic estrogen estrogen action termed xenoestrogens. It has been postulated that the origin of these disorders occurs in fetal life during the delicate process of the development of reproductive system. For these reason, it is important to know how the estrogens and xenoestrogens act during the fetal development and to know the mechanism by which these compounds exert their effects.

The aim of **paper I** was to study the expression of estrogen receptors (ERs) in the embryonic precursors of the adult gametes termed PGCs and to analyze the existence in such cells of intracellular molecular pathways modulable by estrogens. The expression of estrogen receptors α or β (ERα, ERβ) in female and male germ cells has been reported in several species including humans and in some cases, it has been also demonstrated that the ablation of these receptors or in the absence of stimulation by the natural ligand E2 in germ cells (ERKO and ArKO mice) alter some process of their development. The molecular genomic and non genomic bases of such effects remain, however, often to be clarified and the relevance of a direct estrogen effects on germ cells is controversial. It is undoubted that the expression of ERs

renders germ cells a potential direct target of ED. It is mostly important to study the expression and functions of ERs in the embryonic precursors of the adult gametes termed primordial germ cells (PGCs) and to analyze the existence in such cells of molecular pathways modulated by estrogens, since PGCs are not only responsible for establishing the gamete populations but are also the tool for genome transmission to the next generation.

The aim of **paper II** was to investigate the effects of a prototype of xenoestrogens, the pesticide lindane, on primordial germ cells development in the mouse embryo of E12.5 dpc. Numerous reports have shown that lindane adversely affects reproductive function in animals, for example, in adult male rats, chronic exposure to lindane markedly reduces serum testosterone levels, epididymal sperm counts, and sperm motility, and long-lasting effects of lindane on mouse spermatogenesis following in utero exposure have been reported. In adult female mice, rabbits and rats, lindane reduces serum estrogen and progesterone levels, whereas in pregnant mice and minks, it decreases whelping rate and litter size (Beard et al., 1985; Chadwick et al., 1998; Sircar et al., 1990; Srivastava et al., 1993; Sircar et al., 1989). In an in vitro model, the pesticide abolishes oocyte directed follicle organizing activity (Li et al., 1997). Exposure to lindane during the first four days of pregnancy completely prevents implantation in mice, but normal pregnancy results when estrogen and progesterone were coadministered with the compound (Scascitelli et al., 2003). As far as we know, no works have had addressed the effect of lindane on early gametogenesis.

The aim of **paper III** was to verify the presence of functional ERα in embryonic testicular cells using an ERE-and AP1-Luc assay and to evaluate estrogenic activity of putative EDs on mammalian embryonic testis. We and others have reported that mouse embryonic testes from 12.5 dpc onward contain a subpopulation of somatic cells expressing estrogen receptor α (ERα), identifiable mostly as Leydig cells (Greco et al., 1992; Nielsen et al., 2000; Moe-Beherens et al., 2003). This observation marks testes as a possible target for estrogens and estrogenic compounds from early stages of embryo development. Evidence of functional ERα in embryonic testicular cells, however, was lacking and no simple assay exists to evidence estrogenic activity of compounds on such cells.

Introduction

1. The development of gonads in the mouse

In mammals the development of the gonads can be divided into two phases. The **initial phase** is characterized by the emergence of the so-called indifferent, bipotential gonad, or genital ridge, which is identical in males and females. The cell lines that comprise it are bipotential, being able to adopt either the male or female fate. The **second phase** is the development of a testis or an ovary, which is triggered solely by the expression and proper function of the testis-determining gene *Sry*. The indifferent gonads arise as paired structures within the intermediate mesoderm, which lies on either side of the embryo filling much of the coelomic cavity between the limb buds during the first half of development. Within this region, three segments comprising the urogenital ridge are distinguished from anterior to posterior: *1*) the pronephros, which includes the adrenal primordium near its caudal end; *2*) the mesonephros, the central region from which the gonad arises; and *3*) the metanephros, the most posterior region from which the kidney forms.

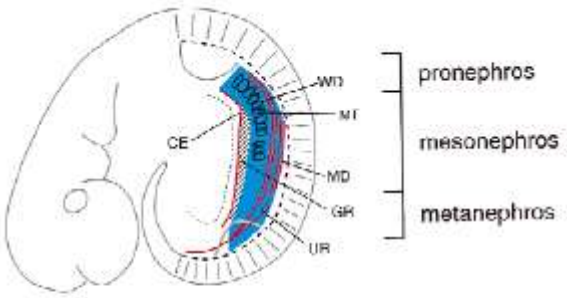


Figure 1. Structure of the urogenital system. Schematic of the mouse urogenital system at 10.5 dpc. Epithelial structures are shown in red, mesenchymal structures are shown in blue, and the striped region denotes the genital ridge. (WD) Wolffian duct (MT) mesonephric tubules; (MD) Mullerian duct; (UB) ureteric bud; (CE) coelomic epithelia. Image from Swain A. and Lovell-Badge R., *Genes Dev.* 1999.

The gonads emerge on the ventromedial surface of the mesonephros at 10.5 days post coitum (dpc). Cells that delaminate from the coelomic epithelium seem to provide one source of cells for the growing genital ridges, while recruitment of underlying cells from the mesonephros to the epithelial population also augments the cell population in the gonadal primordium in males. From the earliest stages of gonad development, the mesonephric tubules can be seen to form continuous bridges to the epithelial cells of the gonad in male and female genital ridges (Karl and Capel, 1995).

In the mouse the gonad initially develops in a non-sex-specific manner, being morphologically identical in XX and XY embryos up until ~12.0 dpc (Fig.2). However, at ~10.5–11 dpc, *Sry* begins to be expressed in the male genital ridge and acts to initiate testis development. In the mouse, *Sry* is expressed in the genital ridge as a wave from anterior to posterior that lasts about a day and a half so that each cell sees it for a few hours only (Hacker et al., 1995).

The action of SRY is therefore thought to trigger differentiation of the Sertoli cell lineage in the testis. Once SRY triggers Sertoli cells they in turn direct the differentiation of the rest of the cell types in the testis. Therefore the decision of sex determination is essentially one of cell fate: SRY triggers Sertoli cell fate in a cell that would otherwise become a follicle cell.

The early mammalian gonad is an undifferentiated primordium composed of bipotential precursor cells that can follow one of two possible fates. Precursors for supporting cells (so named for their role in sustaining and nourishing germ cells in both sexes) and steroid-secreting cells are believed to be present in the early gonad (Merchant-Larios et al., 1993). The supporting cell lineage will give rise to Sertoli cells in the testis and ovary-specific follicle (granulosa) cells in the ovary. These cells surround the germ cells and provide an appropriate growth environment. The steroidogenic cell lineage produces the sexual hormones that will contribute to the development of the secondary sexual characteristics of the embryo. In the male these are the Leydig cells and in the female, the theca cells. The connective cell lineage will contribute to the formation of the organ as a whole. Early testis development is characterized by the formation of testicular cords that contain Sertoli and germ cells, with the Leydig cells excluded to the interstitium. The

connective cell lineage is a major contributor to cord formation as the peritubular myoid cells surround the Sertoli cells and together they lay down basal lamina. The testis is also characterized by rapid and prominent vascularization. Organization of the ovary takes places later than that of the testis and is less structured, with the connective tissue lineage giving rise to stromal cells and with no myoid cell equivalent.

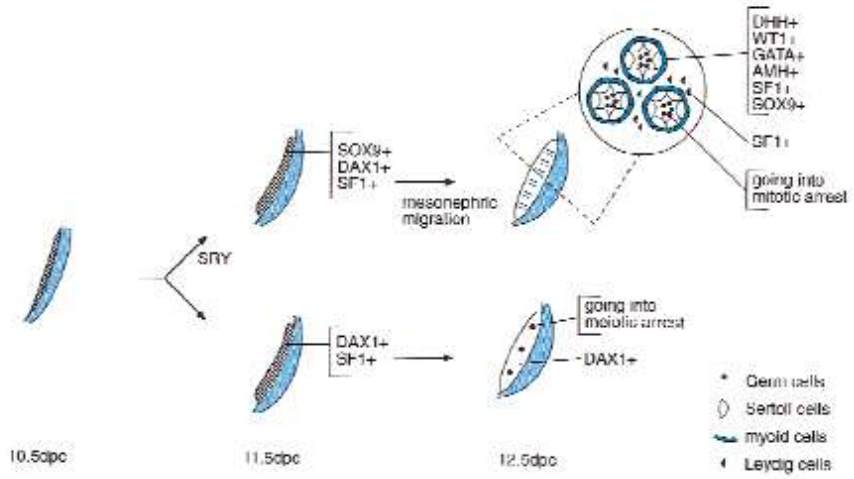


Figure 2. Morphological changes in the gonad during differentiation. Shown are the molecular and structural changes that occur during differentiation of the mouse gonad along the male (*top*) and the female (*bottom*) pathway. The stages of embryo development that are depicted are indicated at the *bottom*. The genital ridge is shown as a striped structure; the mesonephros is shown in blue. Before 10.5 dpc the genital ridge of the embryo is identical between males and females. After the action of SRY, molecular differences in the gonad can be observed, as depicted at 11.5 dpc. Morphological differences between the testis and the ovary can be observed at 12.5 dpc. The structure of testicular cords, which is a consequence of migration of mesonephric cells, is shown as part of the male pathway. The different cell types of the testis and ovary are indicated, as are the genes expressed therein. Myoid cells, indicated in blue, are thought to derive from the mesonephric contribution. The different fate of germ cells between the testis and the ovary is shown. Image from Swain A. and Lovell-Badge R., *Genes Dev.* 1999.

1.1 Origin of Primordial Germ Cells and Their Migration to the Gonadal Ridge

The primordial germ cells (PGCs) do not arise within the genital ridge or the mesonephros but migrate from an entirely separate source. In several mammalian species, due to their characteristic property of positive staining with alkaline phosphatase, from early studies it is possible to trace their formation to the base of the allantois at the posterior end of

the primitive streak. In the mouse results of cell labeling experiments with fluorescent dyes suggest that a population of 45 cells is allocated to the germ line at 7 dpc in the mouse (Lawson and Hage, 1994). At 6–6.5 dpc, the precursors of the PGCs can be found in the epiblast close to the extraembryonic ectoderm, but are evidently not yet restricted to a germ cell fate because they can also form extraembryonic mesoderm. When PGCs are first seen in the mouse at 7 dpc, they are in the region of the forming hindgut. As development proceeds, the hindgut invaginates and the germ cells are swept into the embryo. By 9.5 dpc, PGCs begin to leave the hindgut and pass into the forming urogenital ridges, which are in close proximity at this time. As development proceeds, the hindgut descends into the coelomic cavity and PGCs arriving later must migrate through the dorsal mesentery before entering the developing gonads (Fig. 3).

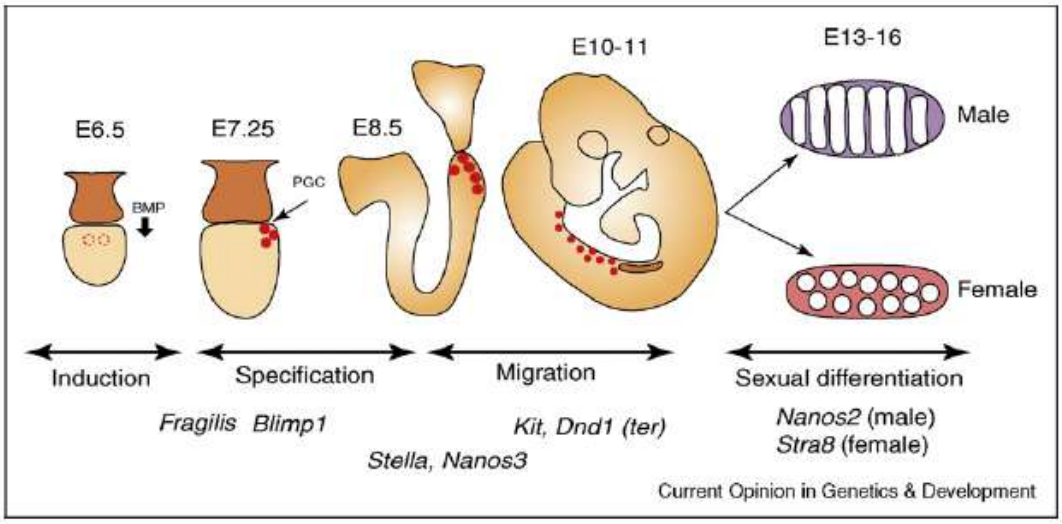


Figure 3. **Schematic representation of germ cell development in the mouse embryo.** The stages of mouse germ cell development and major genes involved in each process are indicated. Primordial germ cells (PGCs) are induced in the epiblast by BMP signaling at E5.5–E6.5. PGCs are specified through the function of Blimp1 by E7.25. PGCs are protected from apoptotic signals during their migration via Kit-mediated signaling, and through the functions of Nanos3 and Dead end1. Once PGCs reach and enter the genital ridge at E11.5, they differentiate according to the somatic sex of the embryo. Nanos2 promotes the male germ cell fate by suppressing the female fate. The locations of the PGCs are indicated by red circles. Image from Saga Y., *Current Opinion in Genetics & Development* 2008.

Survival of the PGCs during migration is dependent on an interaction between the tyrosine kinase receptor c-KIT, which is present on the surface of PGCs, and its ligand, stem cell factor (SCF) also called Kit ligand (Kl), which is produced by the surrounding tissues (reviewed in Bendel-Stenzel et al., 1998). During migration the PGCs also undergo several rounds of cell division to achieve a population of ~3,000 cells by 11.5 dpc, when almost all the PGCs have arrived at their destination. Once inside the gonadal ridge, the germ cells lose their motility and begin to aggregate with one another. They continue to proliferate within the indifferent gonad and maintain their bipotentiality until 13 dpc, where upon germ cells within the male gonad become enclosed within the forming testis cords and enter mitotic arrest as T1 prospermatogonia. In the female, proliferation continues for a short while longer before the germ cells enter meiosis at 13.5 dpc. PGCs thus have the potential to develop either as meiotic oocytes, progressing through the first meiotic prophase and arresting in diplotene just after birth, or as prospermatogonia, mitotically arrested in G1/G0 until a few days after birth, when they resume proliferation (Hilscher et al., 1974; MacLaren, 1995). This developmental switch, which has occurred by 13.5 dpc, is dependent on the sex of the somatic cells in the gonad, rather than the chromosomal sex of the PGCs: XY PGCs can develop as oocytes in female embryos, and XX PGCs can develop as prospermatogonia in male embryos (Palmer and Bourgoyne, 1991).

1.2 Testis Differentiation

Testis differentiation is induced by the expression of *Sry* in a subset of somatic cells that are induced to differentiate into Sertoli cells. Sertoli cells are believed to act as the organizing center of the male gonad and orchestrate the differentiation of all other cell types (Fig 4).

Sertoli cells

Sertoli cells are somatic cells that associate with germ cells and nurture their development into sperm. They are the first cell type known to differentiate within the gonad from bipotential precursors of the supporting cell lineage and are therefore the first indicator that the gonad has passed from the indifferent stage into testis development. Once Sertoli cell fate is triggered by SRY, genes involved in Sertoli cell function become activated. One of these genes is *Amh*. Its product, AMH induces the regression of the Mullerian ducts, which in the female give rise to the oviducts and uterus. Sertoli cells polarize, aggregate around germ cells and reorganize the gonad into two compartments: the tubular testis cods composed of Sertoli and germ cells, and the interstitial space between the cords. Peritubular myoid cells surround Sertoli cells and cooperate to deposit the basal lamina at the periphery of tubule structure. A central role of Sertoli cells is to sustain germ cells during development and later during spermatogenesis. They do so by forming close cell–cell contacts and providing factors involved in growth and differentiation. A candidate factor involved in cell–cell interactions between Sertoli and germ cells is *Desert hedgehog* (*Dhh*), a member of the hedgehog family of molecules that signal at close range.

Peritubular myoid cells

One of the three cell types that migrate from the mesonephros into the male gonad is the peritubular myoid (PM) cell. These cells form a single layer of flattened cells surrounding the Sertoli cells, circumscribing the testis cords. They are thought to have two main functions: *1*) to contribute structurally to the formation of the testis cords in conjunction with Sertoli cells and *2*) to promote the movement of mature sperm through the seminiferous tubules of the adult testis for export to the seminal vesicles, a function

mediated by their smooth muscle-like character. PM cells express α-smooth muscle actin (α-Sma) and desmin. PM cells represent the only cell type in the testis so far for which no counterpart can be identified in the ovary. This might be due to their origin from immigrating cells from the mesonephros, which only occurs in an XY gonad after the expression of *Sry* (Capel et al., 1999; Martineau J et al., 1997).

Leydig cells

Within the second compartment of the testis, the interstitium, steroidogenic Leydig cells differentiate. These cells secrete a hormone that plays a role in establishing and maintaining the secondary male sex characteristics. Leydig cells often lie in clusters close to blood vessels, in line with their steroidogenic role. In mammals there are two types of Leydig cells. The fetal Leydig cells originate, at least in part, in the mesonephros, and are responsible for the production of androgen for the fetal masculinization; these cells probably degenerate postnatally. The adult Leydig cells, which differentiate after birth, appear to be unrelated to their fetal counterparts. Studies indicated that they arise from undifferentiated precursor cells that are part of the mesenchymal cells of the interstitium (Hardy, 1993). The origin and roles of Leydig cells are discussed comprehensively in Chase 1982.

Vascular and other interstitial cells

Although Leydig cells are often considered the main component of the testicular interstitium, probably because of their essential and obvious male-specific endocrine roles, several other interstitial cell types can be found. These include endothelial cells, fibroblasts, and blood-derived cells such as macrophages, lymphocytes, plasma cells, monocytes, and mast cells. Endothelial cells, alongside PM and Leydig cells, represent a third cell type that migrates into the testis from the mesonephros (Martineau et al., 1997). They form the male-specific vasculature with the prominent coelomic vessel on the surface of the gonad and side branches in between the testis cords.

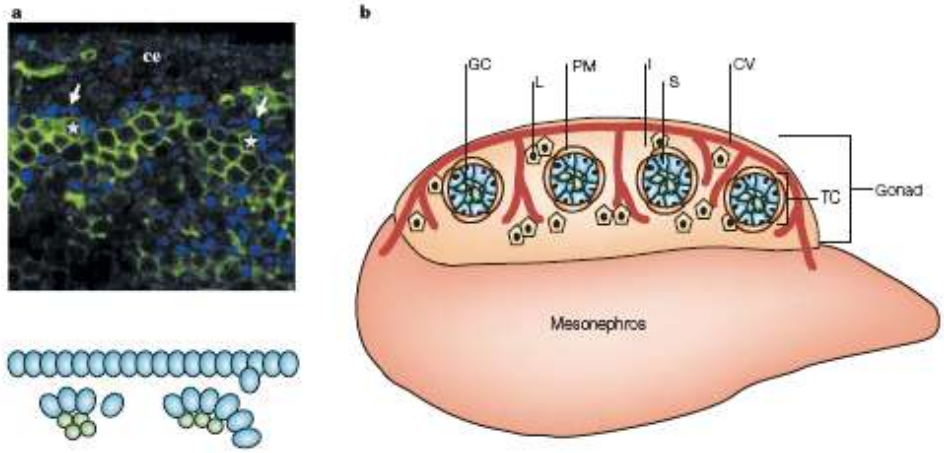


Figure 4. Compartmentalization of the testis. a At the earliest stages of testis organogenesis (11.75–12.0 days post coitum(dpc), Sertoli cells (stained with SF1 antibody; blue) polarize and begin to aggregate around clusters of primordial germ cells (stained with PECAM antibody; asterisk) to initiate development of testis cords. ce, coelomic epithelium. **b** Between 11.5–12.5 dpc, the cells of the testis are organized into two functional compartments: testis cords (TC) and the interstitial space (I) outside the cords. Within testis cords, Sertoli cells (S; blue) surround germ cells (GC; green). A basal lamina is deposited between Sertoli cells and peritubular myoid cells (PM). The interstitial compartment contains Leydig cells (L; yellow) and the coelomic vessel (CV; red), with branches that extend between cords. Image from Brennan J. and Capel B., *Nature Reviews* 2004.

2. Estrogens and Estrogen Receptors

Estrogens (U.S., otherwise **oestrogens**) are a group of steroid compounds, named for their importance in the estrous cycle, and functioning as the primary female sex hormone. The three major naturally occurring estrogens in women are estradiol, estriol, and estrone. Estradiol, like other steroids, is derived from **cholesterol**. After side chain cleavage and utilizing the delta-5 pathway or the delta-4 pathway androstenedione is the key intermediary. A fraction of the androstenedione is converted to testosterone, which in turn undergoes conversion to estradiol by an enzyme called aromatase. Estrogen is produced primarily by developing follicles in the ovaries, the corpus luteum, and the placenta. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) stimulate the production of estrogen in the ovaries. Some estrogens are also produced in smaller amounts by other tissues such as the

liver, adrenal glands, and the breasts, fat cells are active to convert precursors to estradiol, and will continue to do so even after menopause. They are usually present at significantly higher levels in women of reproductive age. They promote the development of female secondary sex characteristics, such as breasts, and are also involved in the thickening of the endometrium and other aspects of regulating the menstrual cycle. Also men produce estrogens that are present in adult where they regulate certain functions of the reproductive system important to the maturation of sperm (O'Donnell et al., 2001).

Estradiol enters cells freely but the pleiotropic effects of estrogens are transduced trough multiple ER receptor subtypes as ERα/β or GPR30 (G-protein coupled receptors-30) that have multiple subcellular locations (Filardo and Thomas, 2005; Watson and Gametchu, 2003). These receptors inhabit nuclei and cytoplasm, plasma membranes or perimembrane spaces (Clarke et al., 2000), endoplasmic reticulum (Revankar et al., 2005) and mitochondria; they also sometimes change locations or arrangements within their locations, depending upon liganding or other circumstances (Song et al. 2002).

2.1 ERα and ERβ

The two mammalian ERs exhibit modular structures characteristic of the nuclear receptor superfamily. The two receptor are not isoforms of each other, but rather different proteins encoded by separate genes located on different chromosomes (10 and 12 respectively in mouse, 1 and 6 in rat, and 6 and 14 in humans). Hormone activated estrogen receptors form dimers, and since the two forms could be are coexpressed in many cell types, the receptors may form ERα (αα) or ERβ (ββ) homodimers or ERαβ (αβ) heterodimers. The ER proteins are each composed of six functional domains labeled A–F, a signature characteristic of the entire superfamily (Fig. 5).

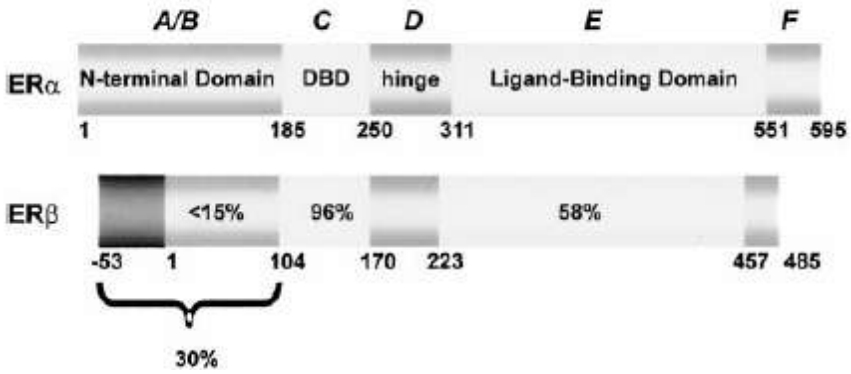


Figure 5. Comparison of the structure of ERα and ERβ proteins. The functional domains A–F and the percentage homology of ERβ compared with ERα are shown. Indicated are the N-terminal domain (A/B domain), the DNA binding domain (DBD) (C domain), the hinge region (D domain), the ligand-binding domain (E domain), and the C-terminal region (F domain). The two potential start sites on ERβ are designated 253 and 11.Image from O'Donnell et al., *Endocrine Reviews* 2001.

The N'-terminal A/B domain is the least conserved among all members and demonstrates only 17% homology between the two ERs. It contains the activation function 1 **(AF1)** region, which is one of two regions critical for the transactivation function of the members of the receptor family. By contrast, the **C** domain is the most highly conserved region, being the DNA binding domain that contains the zinc-finger motifs. The **E** domain, or ligand-binding domain, is modestly conserved throughout the superfamily and confers ligand specificity on the members. Conservation of amino acid sequence in this region is 60% between the ERα and ERβ; however, each binds estradiol with about equal affinity, although the relative binding of other ligands differs substantially between them (Kuiper et al., 1997; Kuiper et al.1998). The E domain also contains the major dimerization surface of the receptors, and the second transactivation function, activation function 2 (AF-2), is also located in this region of the C'-terminus. Transcription of the mouse ERα gene in vivo leads predominantly to a single transcript of approximately 6.3 kb, encoding a protein of 599 amino acids. Initial studies of the rodent ERβ transcript indicated it was substantially shorter than the ERα, namely 485 amino acids (Kuiper et al., 1996). This is largely due to a significantly shorter N'-terminal region . Due to alternative RNA splicing, several ER isoforms are known to

exist. At least three ERα and five ERβ isoforms have been identified. The ERβ isoforms receptor subtypes can only transactivate transcription when a heterodimer with the functional ERß1 receptor of 59 kDa is formed. The ERß3 receptor was detected at high levels in the testis. The two other ERα isoforms are 36 and 46kDa (Nilsson et al., 2001). Only in fish, but not in humans, an ERγ receptor has been described (Hawkins et al., 2000). There is considerable tissue specificity in the expression of ERα and ERβ. Thus, ERα is the dominant species expressed in uterus, liver, adipose, skeletal muscle, pituitary, and hypothalamus, whereas ERβ is the major form in ovary and prostate, as well as other regions of the brain including the limbic system, cerebellum, and cerebral cortex (Couse et al., 1997).

2.2 Estrogen related receptors

At the superfamily of the nuclear receptors belong putative receptor molecules for which no known ligands are identified and these are classified as orphan nuclear receptors. Estrogen-related receptors belong to a subfamily of such orphan nuclear receptors, and comprise of three members, ERR-α, -β, and -γ (Giguère et al., 1988 and Giguère, 2002). These ERR proteins are, as their names indicate, closely related to the ERs in their structures and bind to the estrogen response elements, but they are not activated by estrogen (Giguère, 1988). Three ERR proteins display a high degree of structural similarities within binding domains for their ligand and DNA, suggesting that they would bind to similar ligands and targets.

2.3 GPR30

GPR30 is an orphan member of the G-protein-coupled receptor superfamily, that has been reported to trigger rapid signaling by estrogen. GPR30 is localized mainly into endoplasmic reticulum and binds E2 with nanomolar affinity (Filardo et al., 2000; Revankar et al., 2005). The biological relationship between GPR30 and conventional ERs is currently unknown and further studies are required to determine the relative contribution of either or

both of these pathways to estrogen signaling. Many studies have indicated that this receptor through a cross-talk between ERα activate the rapid Egfr/Erk/Fos pathway that in turn stimulate mouse spermatogonial cells (GC-1) or ovarian cancer cells to proliferate (Sirianni et al., 2008; Albanito et al., 2007). It is possible however that the rapid E2 signaling is mediated by a complex network of proteins that consists of conventional steroid receptors and other steroid binding proteins such as GPR30 (Cheskis, 2004).

2.4 Membrane estrogen receptors

Classical steroid receptors, localized in the cytosol and/or nucleus, traditionally mediate their primary effects at the genomic level. In recent years, a large number of reports have described membrane-associated estrogen receptors, either similar to or distinct from the classical nuclear estrogen receptors (Acconcia et al., 2004; Razandi et al., 2003). These receptors have been postulated to mediate aspects of cellular estrogen function, including traditional genomic (transcriptional) signaling as well as novel non-genomic (rapid) signaling.

Although the majority of ERα is localized in the nucleus, there is evidence that a small fraction of the receptor is localized at or near the cell membrane in either the presence or absence of E2. While the precise mechanism(s) are currently unknown, it has been proposed that ERα translocation to cell membrane is mediated by its interactions with membrane proteins. Candidate interacting proteins include **caveolin-1/-2** and the 110-kDA caveolin-binding protein–striatin. Caveolae are specialized regions of the plasma membrane that assemble and organize signaling protein complexes (Shaul, 1998). **Striatin** is a calmodulin-binding member of the WD-repeat family of proteins. It has been reported to anchor ERα to the cell membrane, and to serve as a scaffold for the formation of an ERα–Gai complex, which is critical for E2 activation of eNOS (Lu et al., 2004). It has been also proposed that ERα can be targeted to the cell membrane by the adaptor protein **Shc** (Pelicci et al., 1996). The SH2 domain of Shc can directly interact with the N-terminal part of ERα (Song et al., 2002). Recently another membrane adaptor protein the p130Cas (Crk-

associated substrate) has also been reported to interact with ERα-cSrc complex in T47D breast cancer cells and to potentiate the E2 activation of Src (Cabodi et al., 2004). Finally, palmitoyl-acyl-transferase (**PAT**)-dependent S-palmitoylation of ERα was recently reported to promote Erα association with the plasma membrane and interaction with caveolin-1 (Acconcia et al., 2004, 2005). Furthermore, cystine 447-mutated ERα did not stimulate activation of MAP and PI3 kinases (Acconcia et al., 2005). A terminally truncated 46 kDa variant of ERα has been found to be preferentially palmitoylated and enriched in plasma membrane of several cell types (endothelial, osteoblasts, and MCF-7 cells) (Denger et al., 2001).

In conclusion, several membrane proteins have been identified that interact with classical receptors and influence their non-genomic action. However, the precise role of these proteins in receptor regulation of the cell signaling remains to be further investigated. It is possible that the composition of ER complexes at the plasma membrane is cell context dependent, which may potentially explain the cell type selectivity of non-genomic action.

3. Mechanisms of estrogens action

3.1 Nuclear genomic action

ERE-dependent transcriptional regulation

The classical mechanism of steroid hormone action involves nuclear interactions of intracellular ERs receptors, which are either **cytoplasmic** or **nuclear**. Binding of hormone to ER releases the receptor from an inhibitory complex with HSPs (Heat Shock Proteins) and triggers conformational changes that allow ER to bind the responsive elements in the target gene promoters (Fig.6). Subsequently, the receptor-ligand complex binds to the a 15-bp palindromic **ERE** (Estrogen Response Element) located in the target gene promoters, and stimulates gene transcription. The ERE is a 13 base pair inverted repeat sequence (GGTCAnnnTGACC), and the ER binds as a dimer, with one ER molecule contacting each 5 base pair inverted repeat (Klinge et al., 2001). Maximum transcriptional activity requires the concerted actions of the ligand-independent AF1 domain and the ligand-dependent AF2 domain.

The transcriptional activity is also affected by a number of regulatory cofactors including chromatin-remodeling complexes, coactivators, and corepressors. Coactivators generally do not bind to the DNA but are recruited to the target gene promoters through protein-protein interactions with the ER. Examples of ER coactivators include, members of the **p160/SRC** (Steroid Receptor Coactivator) family: SRC1/NCoA1 (Nuclear Receptor Coactivator-1); NCoA2; NCoA3/AIB1/TRAM1/RAC3; the cointegrators: CBP (CREB-Binding Protein) and p300; the family of **CITED** (CBP/P300-Interacting Transactivator,With Glu/Asp-Rich Carboxy-Terminal Domain) proteins. Corepressors like NCoR (Nuclear Receptor Co-Repressor) and MTA1 (Metastasis Associated-1) protein have been implicated in the transcriptional silencing. In addition, a few bifunctional coregulators such as PELP1 (Proline Glutamic Acid-Rich Nuclear Protein) also exist that can act both as coactivators and corepressors of ER. It is the relative balance of receptors, coactivator and

corepressor proteins, which is a critical determinant of the ability of this classical pathway to initiate responses. Since the relative concentrations of these molecules is cell specific, sex steroid hormones can have vastly different functions in different tissues of the same organism

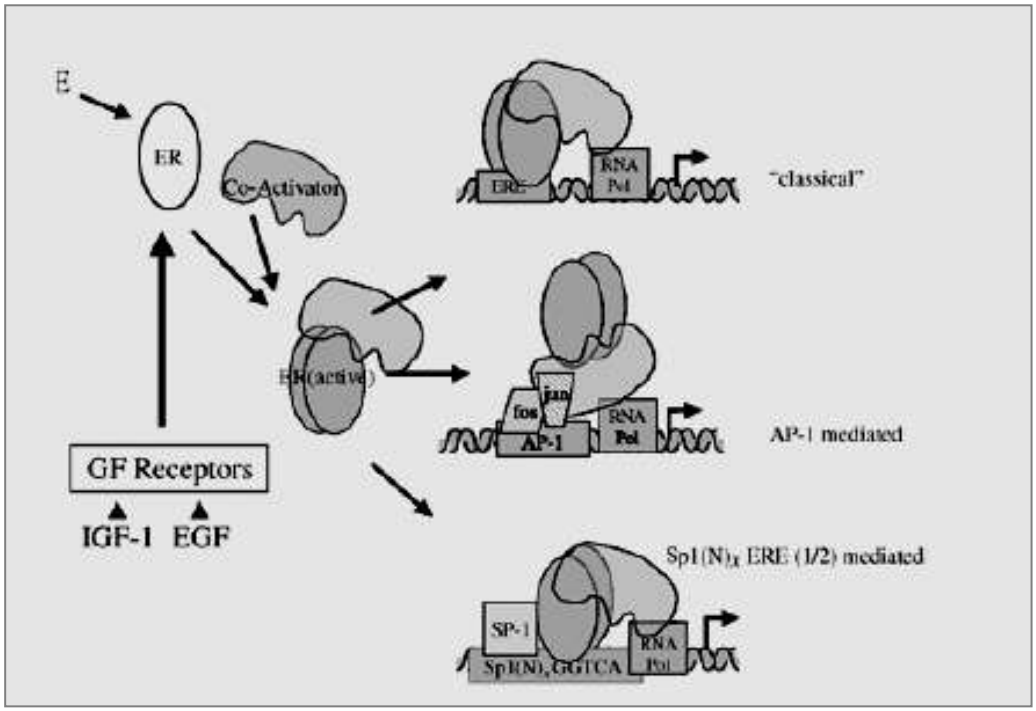


Figure 6. Mechanism of ER mediated transcription ER-mediated transcription is initiated following E2 binding or ligand-indipendent activation resulting from growth factor receptor pathway signalling and cross-talk with ER. Once activated, ER dimers recruit coactivators and can mediated transcription of genes via direct binding to EREs in target genes (classical mechanism). Alternatively, ER can recruit coactivators to an AP-1 complex (AP-1 mediated mechanism). Finally, ER can interact with promoters containing Sp1 binding sequences and ERE-half sites ($Sp1(N)_x$ ERE(1/2)mediated). Image from Hewitt and Korach. *Reviews in Endocrine and Metabolic Disorders* 2002.

ERE-indipendent Genomic action of ER

In recent years, mechanisms of gene regulation by ERs that deviate from this ‘‘classical model’’ have also been described. These include gene regulation by ERs that does not involve direct receptor binding to DNA, but rather via ERs participation in the formation of the pre-initiation complex via protein–protein interactions, such as the **AP1 complex** (Webb et al., 1999), as in the case of the collagenase and IGF-I genes. First, binding of Jun and Fos to the AP-1 site is needed for ER action, and ER appears to increase the intrinsic transcriptional activity of Jun:Fos when bound to the site (Fig.6). Estrogen regulation is also seen in genes such a c-FOS and TGFa which lack a full ERE sequence. This regulation is mediated by an interaction between ERα and SP1 proteins, which bind ERE-half sites and GC rich sequence, respectively, in the regulatory regions of these genes. ERα interacts with Sp1 protein to transactivate genes through binding Sp1(N)xERE or Sp1(N)xERE half-site (1/2) motifs where both ERs and Sp1 bind DNA elements (Safe S. 2001).

3.*2 Extranuclear/Nongenomic Action of Estrogens*

In addition to transcriptional regulation, which occurs on a time scale of hours, estrogen also mediates cellular effects with response times from seconds to minutes. These rapid non-genomic estrogen signaling events include the generation of the second messengers Ca^{2+}, cAMP, and NO, as well as activation of receptor tyrosine kinases, such as EGFR and IGF-1R, and protein/lipid kinases (e.g. PI 3-kinase, Akt, MAPK family members, Src family members, and PKA/PKC). In many reports, the estrogen-responsive receptor is proposed to be ER itself (either α or β), or a modified form of the protein (Acconcia et al., 2004) (Fig.7). Complexes between the classical **ERs and G proteins** (Navarro et al., 2003) as well as with PI3 kinase (Simoncini et al., 2003) have been described. Recently, ER associations with plasma membrane Gi proteins have been reported to mediate NO production and cAMP inhibition (Navarro et al., 2003). The nature of the upstream receptor targets remains to be better established.

Multiple lines of evidence suggest that activation of the tyrosine kinase **cSrc** represents one of the initial steps in ERα-mediated cell signaling in MCF7 cells (Migliaccio et al., 2002). cSrc can be initially activated either by dephosphorylation of the C-terminal inhibitory phosphotyrosine site (Tyr 529) or in oncogenic variants by loss of the C-terminal tail), or by binding of high affinity ligands to the SH2 or SH3 domains. These domains are modular polypeptide units that mediate protein–protein interactions and are found together on many proteins, suggesting that their activities can be coordinated and that they can cooperate in Src regulation (Cohen et al., 1995). After this initial activation, Src autophosphorylation loop leads to phosphorylation in Tyr 418 and stimulate Src activity (Superti-Furga et al., 1995).

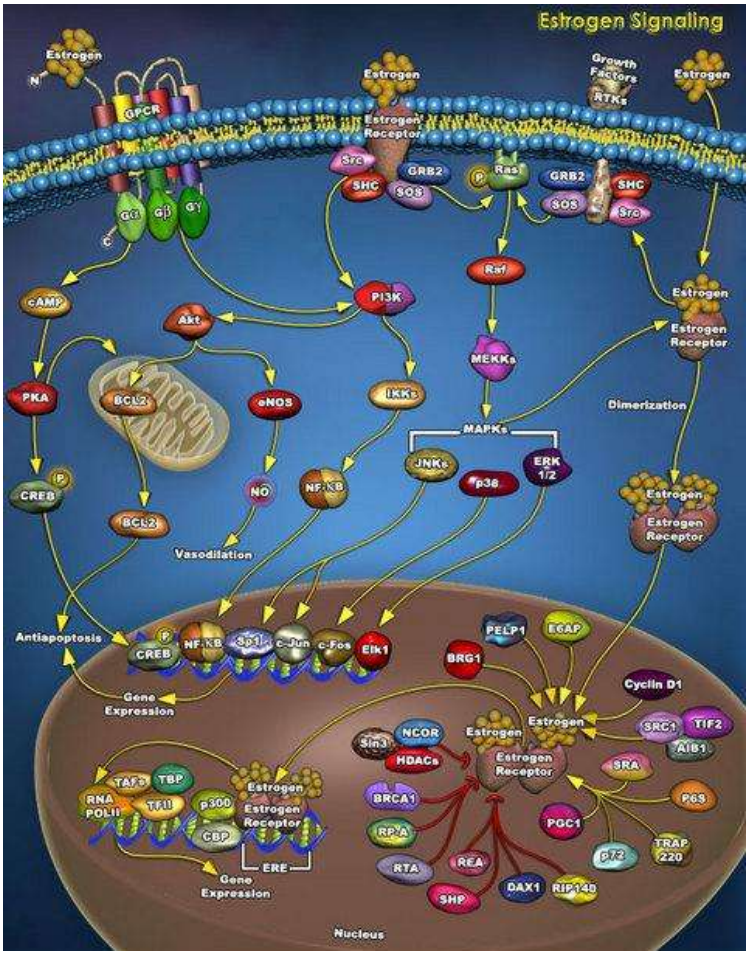


Figure 7. Signaling by estrogens mediated by a complex interface of direct control of gene expression (genomic action) and by regulation of cell signalling/phosphorylation (non genomic action).

Recently, an adaptor protein Modulator of Nongenomic Action of Estrogen Receptor (MNAR) has been identified that is required for E2 induced ERα activation of cSrc and downstream MAP kinase pathway (Wong et al., 2002). It has been recently demonstrated that in MCF7 cells treated with E2, endogenous MNAR, ERα and cSrc also interacted with p85, the regulatory subunit of the PI3 kinase. Further, ERα-MNAR activation of cSrc, led to MNAR phosphorylation on Tyr 920 which was required for its interaction with SH2 domain of p85 and activation of the PI3K/Akt pathway. Existing data indicate that MNAR is a scaffold, which is promoting receptor binding to Src and stabilizing ERα-Src complex.

Crosstalk between **growth factors** and ERs takes place in both nuclear and cytoplasmic compartments. Both, EGF and IGF can also activate ERα transcriptional activity in presence of E2. E2 may rapidly activate the two main signaling cascades coupled to the IGF-I and the EGF receptors: the PI3K/Akt and the Src/MAPK signaling pathways. Initially an indirect mechanism of ERα activation of epidermal growth factor receptor (EGFR) has been proposed to explain these effects. According to this hypothesis, ERα bound to caveolin-1 in the cell membrane could interact with a Gprotein- coupled receptor, which in response to estrogen- or tamoxifen-binding may directly or indirectly interact with and activate specific G proteins. The subsequent activation of cSrc leads to activation of matrix metallo-proteinases, which in turn cleave heparin-binding epidermal growth factor (EGF) from the membrane. This form of EGF then binds to surface EGFR in an autocrine or paracrine manner to activate the receptor and its downstream kinases including ERK 1/2 MAPK and Akt (Levin, 2002; Levin 2003). Recent evidence however indicates that ERα and Src may play a direct role in EGF activation. Direct interaction has also been documented for ERα and IGF receptor both in vitro and in vivo (Mendez et al., 2003). The interaction was coincident with the increase in tyrosine phosphorylation of IGF-I receptor, suggesting a possible causal relationship (Mendez et al., 2003).

One of the best-characterized extranuclear actions of estrogens is the rapid activation of the **Ras/Raf/MAPK** pathway. In neuronal cells, E2 rapidly triggers Erk 1/2 activation, leading to cFos gene expression. E2 activated growth of human colon carcinoma-derived

Caco-2 cell was found to be mediated through rapid and reversible stimulation of the cSrc and cYes, and subsequent activation of ERK1 and ERK2 kinases. E2-mediated stimulation of Ras/Raf/ERK pathway promotes MCF7 cell proliferation (Migliaccio et al., 1996). The MAPK pathway is involved in the control of many fundamental cellular functions that include cell proliferation, survival, differentiation, apoptosis, motility, and metabolism. Another well-characterized and biologically important action of E2 is the acute effect on blood vessels to stimulate vasodilatation and protect against vascular injury. This action is mediated by a subpopulation of ERα in plasma membrane of endothelial cells through the activation of eNOS and the stimulation of NO production via the PI3 kinase/Akt signaling pathway. One of the important down stream targets of PI3K is the threonine-serine kinase Akt/ protein kinase B. Activation of PI3K/Akt by E2 has also been shown to be important in breast cancer cells in mediating E2- stimulation of cell cycle progression (Castoria et al., 2001) and inhibition of apoptosis. Many cell-signaling pathways converge upon and regulate the phosphorylation status and hence activity of multiple transcription factors, which affects gene expression. Several examples of this mode of regulation have been reported, including ERα-dependent E2 regulation of the c-fos gene mediated by Src/MAP and Src/PI3 kinase pathways converging on Elk-1 and SRF, respectively; E2 regulation of cyclin D1 mediated by PI3K/Akt pathway and E2 regulation of the Egr-1 gene mediated by MAP kinase activation of SRF.

4. Estrogens and development of mouse gonads

4.1 Estrogen Receptor KO mice

The advent of gene deletion techniques has allowed the generation of mice lacking ERα (Couse and Korach, 1999), ERβ (Krege et al., 1998) or both ERα and ERβ (Couse and Korach, 1999), as well as mice that lack the ability to synthesize estrogens due to deletion of the aromatase gene (Fisher et al., 1998). Characterization of the phenotypes exhibited by these models exert that both sexes of the αERKO mice are infertile, whereas only the

βERKO female has shown impaired fertility. In the **male** αERKO mice, infertility is due to deficits at several points in the reproductive process, including severe reduction in sperm numbers and lack of sperm function, as well as abnormal sexual behavior. The seminiferous tubules of the αERKO testes show progressive dilation that is accompanied by degeneration of the seminiferous epithelium (Fig. 8). In contrast, the testes of the βERKO mice appear normal (Fig. 8), and produce sufficient and functional sperm to allow fertility, resulting in production of offspring in mice examined to date. Therefore, αER appears to be more critical than ERβ in mediation of the estrogen actions necessary for maintenance of healthy testicular structures and the somatic cell function required for successful sperm maturation. Double mutant for both receptors α and β (αβERKO) are infertile and show phenotype and alterations of reproductive tract functions comparable to αERKO (Couse et al., 1999).

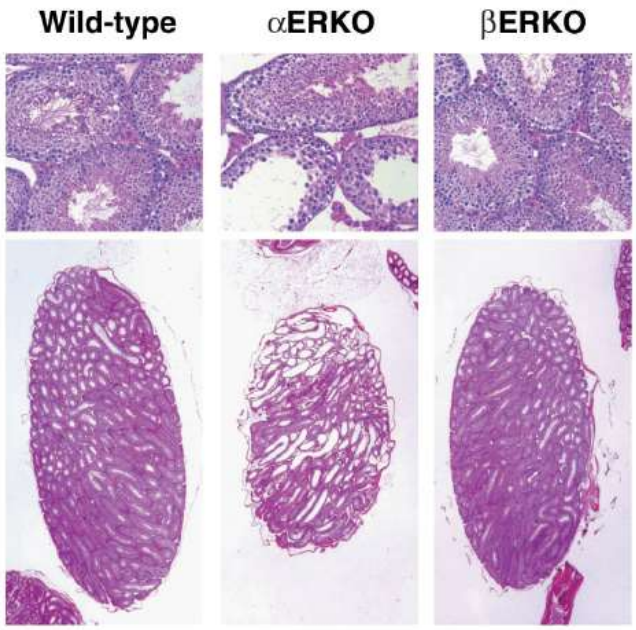


Figure 8. Pathology of adult αERKO and βERKO testes. The wild-type and βERKO are indistinguishable, while the αERKO testis shows degeneration of the testicula structures. Image from Hewitt S.C. et al., *Breast Cancer Res* 2000.

Normally, the **female** rodent reproductive tract grows and matures in response to cycling ovarian hormones, including estradiol. The ovarian phenotypes are a major component of the infertility in the αERKO mice and the subfertility in the βERKO mice. The αERKO female does not ovulate, while the βERKO female is subfertile with reduced litter numbers and smaller litter sizes compared with wild-type littermates. Interestingly, although both ERα and ERβ are detected in the ovary, their localization differs with ERβ in the granulosa cells and ERα in the theca and interstitial cells of the ovary. The hallmark phenotype of the αERKO female is the enlarged hemorrhagic cystic ovary (Fig. 9), although the prepubertal αERKO ovary looks similar to its wild-type littermate. This phenotype begins to develop progressively as the animal matures and is apparently due to a lack of estradiol feedback inhibition in the pituitary, which results in chronically elevated LH and subsequent hyperstimulation of the ovary.

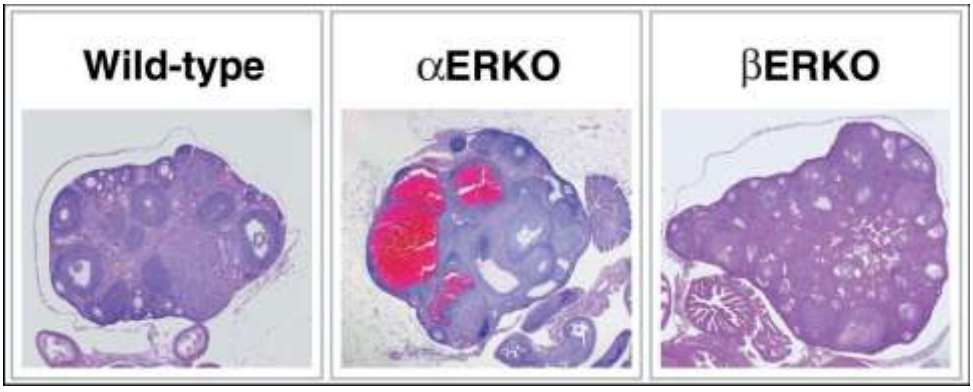


Figure 9. Ovarian pathology of the ERKO mice. Histological analysis of the wildtype ovary shows normal follicular development and indications of ovulation. The αERKO ovary shows large cystic structures and arrested follicle development with no indication of ovulation, while the βERKO ovary shows development of follicles is occurring but with little indication of successful ovulation. Image from Hewitt S.C. et al., *Breast Cancer Res* 2000.

This indicates that ERα is responsible for mediating the LH feedback inhibition in the hypothalamic–pituitary axis. The constant LH stimulation in the αERKO mice results in an abnormal endocrine environment in the αERKO female, with elevated estradiol and

testosterone, and chronic preovulatory basal progesterone levels. The βERKO ovaries produce normal serum levels of estradiol and testosterone, and the circulating serum gonadotropin levels are also normal. However, the βERKO ovaries function suboptimally, as illustrated by the appearance of numerous unruptured follicles following superovulation. Attempts to superovulate the βERKO female results in some ovulation, but the number of oocytes released is reduced compared with wild-type females. A role for ERβ in ovulation is thus indicated, but the mechanism is still being defined. Female αβERKO are infertile, the ovaries exhibit follicle transdifferentiation to structures resembling seminiferous tubules of the testis with postnatal sex reversal (Couse et al., 1999).

4.2 Estrogen receptor expression and role during development of mouse testes

ERα and ERß are present in the testis very early in development and their distribution in various types of testicular cell has been extensively studied in mammals. In the mouse, immunohistochemical data have shown that ER protein is present in the undifferentiated gonad as early as 10.5 dpc (Greco et al. 1992) and is localized mainly in the fetal Leydig cells until birth in rodents(reviewed in O'Donnell et al. 2001). Only one study has shown some staining in the seminiferous cords (Greco et al. 1992). ERß mRNA is detected in the testis as early as 14 dpc in the mouse and is present primarily in the gonocytes, but also in the Sertoli and Leydig cells, as early as 16 dpc in the rat, while in the rat ERß protein is present at 16 dpc in the three main types of testicular cells and it is found exclusively in the gonocytes in the mouse (Saunders et al. 1998). Immunohistochemical analysis has shown that in humans, ERα is not present in the testis but ERß is expressed in germ cells, Sertoli cells, and Leydig cells (Saunders et al. 2001). There are few papers on mouse that describe the role of estrogen during the fetal life. Geraldine Delbès provided the first demonstration, utilizing the mice inactivated for ERα and ERβ (Delbès et al. 2004, Delbès et al., 2005), that endogenous estrogens physiologically regulate testicular development in a negative manner during fetal and neonatal life by controlling the two main functions of the testis, gametogenesis and steroidogenesis. In fact, the inactivation of the ERß gene induced a 50%

increase in the number of gonocytes observed 2 and 6 days after birth due to an increase in the proliferation and a decrease in the apoptosis of these cells, with no change in Sertoli cell or Leydig cell number. The inactivation of the ERα caused fetal Leydig cell hypertrophy and induced higher levels of StAR, P450scc, and P450 c17 mRNA in such cells. These data clearly show that endogenous estrogens inhibit some important process of testicular development and function during fetal and neonatal life. In summary ERß is involved in the control of germ cell proliferation/apoptosis, consistent with its location within the seminiferous cords, whereas ERα is mainly present in the fetal Leydig cells and regulates steroidogenesis.

5 Endocrine Disrupters

During the last decades, epidemiological studies in many countries have shown trends of increased incidences in disorders of the male reproductive system which include testicular cancer, cryptorchidism, hypospadias/intersex, and subfertility (Toppari et al., 1996). These four reproductive disorders have been suggested to be symptoms of one underlying entity, the testicular dysgenesis syndrome (TDS) (Skakkebaek et al., 2001) (Fig. 10). It is currently thought that TDS is caused by changes occurring during the fetal period, because the origins of the four disorders can be traced during the fetal development. For example, it is know that cryptorchidism results from the abnormalities in the production or activity of Insl3 or the androgens regulating the transbdominal descendent of the testes (Kubota et al., 2002). Hypospadias results from a defect in androgens production or action during fetal development. In effect, Hypospadia is caused by a defect on production of testosterone and how consequence of its action. The etiology of the testis cancer remain unclear, but there is considerable evidence to suggest that it originates, at least some type of germ cell tumors such as teratoma, early in development (Skakkebaek et al., 2001) when gonocytes would normally have differentiated into spermatogonia.

The increased incidence of TDS cannot explained by genetic factors alone, and instead environmental and life-factors have been suggested as causes of TDS, like exposure

to environmental chemicals that possess endocrine-disrupting activity (Toppari et al., 1996; Skakkeaek et al., 1998).

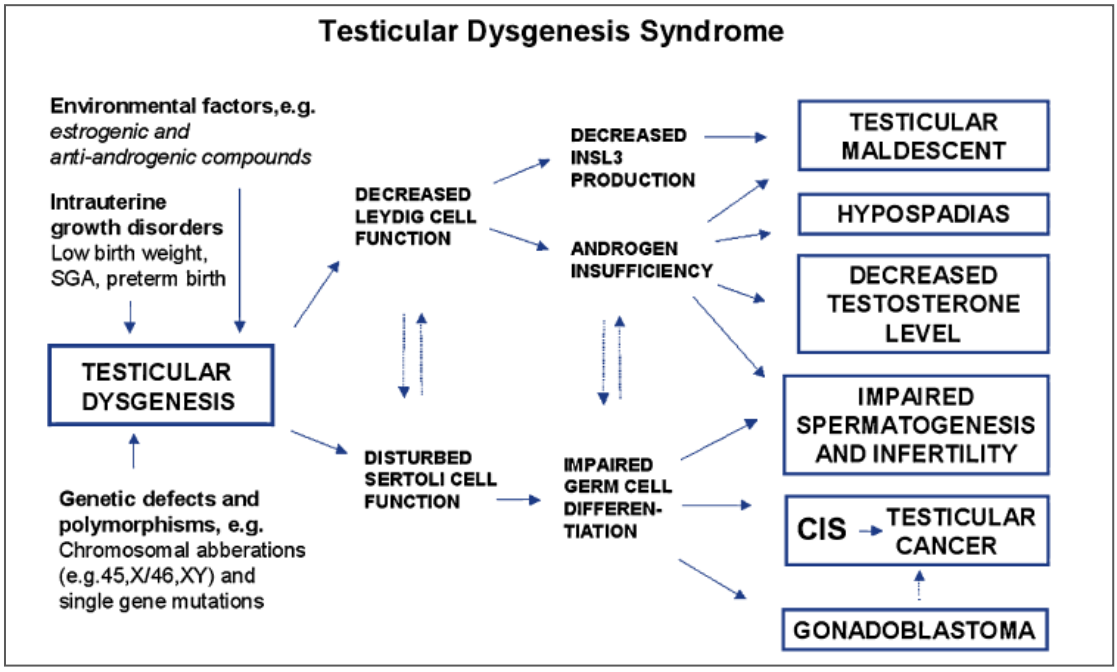


Figure 10. Schematic illustration of the pathogenesis and aetiology of the testicular dysgenesis syndrome (TDS). Image from Skakkebaek et al, *Human reproduction* 2001

The **Endocrine disrupters** are exogenous substances that act like hormones in the endocrine system and disrupt the physiologic function of endogenous hormones. Endocrine disrupting compounds encompass a variety of chemical classes, including hormones, plant constituents (phytoestrogens), pesticides, compounds used in the plastics industry, in consumer products, and other industrial by-products and pollutants (Table 1). EDs that mimic the action of natural estrogens are termed xenoestrogens. The most common mode of exposure to EDs is through dietary sources. Human ingestion of phytoestrogens, for example, is quite significant, especially in soy supplemented diets. The most important of these compounds in terms of human consumption are the isoflavones (**genistein** and

daidzein), found mainly in soy products, but also present in fruits and nuts. Phytoestrogens such as these are increasingly marketed as over-the-counter, natural products, for use as an alternative to hormone replacement therapy in post-menopausal women. **Zearalenone** (6-[10-hydroxy-6-oxo-trans-1-undecenyl]-Bresorcyclic acid lactone) is a mycoestrogen biosynthesized by the fungi Fusarium graminearum, Fusarium culmorum, Fusarium equiseti, and Fusarium crookwellense (Bennett and Klich, 2003). These fungi are commonly found in cereal crops and it has also been patented as an oral contraceptive (Bennett and Klich, 2003). **Bisphenol-A** (BPA) is an industrial monomer used in production of polycarbonates and epoxy resins. There is considerable potential for human exposure to this compound because traces of it are known to leach from the lining of food cans, plastic ware, and from dental sealants. BPA is less likely to bioaccumulate than some xenoestrogens because it is readily metabolized through glucuronidation followed by excretion. Nonetheless, there have been numerous in-vitro and *in-vivo* studies of the estrogen-like effects of BPA, which illustrate its potential for endocrine disruption, and adverse effects on development. **Phthalates** called "plasticizers," are a group of industrial chemicals used to make plastics like polyvinyl chloride (PVC) more flexible. The most widely-used phthalates is the di-2-ethyl hexyl phthalate (DEHP). Recently it has been demonstrated that phthalate, when administered orally, are rapidly hydrolyzed in the gut and other tissues to produce the corresponding monoesters mono-(2-ethylhexyl) phthalate (**MEHP**), one of the metabolites of DEHP, showed the most potent testicular toxicity. Some of the more prevalent synthetic estrogens in the environment include DDT metabolites and polychlorinated biphenyls (PCBs) or organohalogenated pesticide like the γ-isomer of hexachlorocyclohexane lindane**. Lindane** (γ-HCC) has been largely used as an insecticide and disinfectant in agriculture and entered also in the composition of some lotions, creams and shampoos used against parasites (lice and scabies). These organochlorines, for their physical and chemical characteristics, are known to accumulate and persist in biological matrices, human blood, adipose tissues and milk (termed bioaccumulation).

In table 2 and 3 are reported some of the *in vitro* and *in vivo* effects exerted by estrogens and estrogenic compound in rodents fetal testis .

Table.1. Chemicals with endocrine disrupting activity. Compounds used in our studies are indicated.

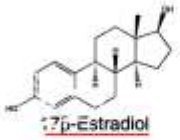
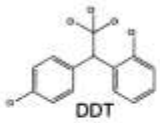
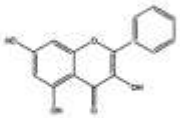
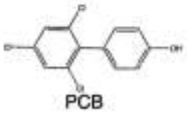
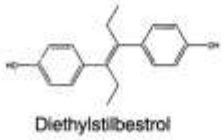
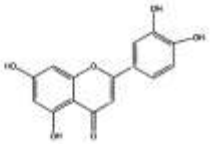
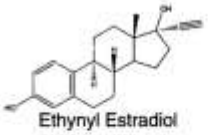
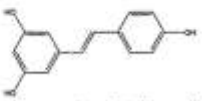
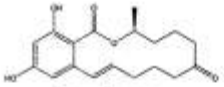
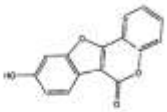
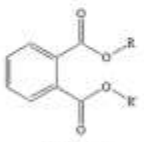
Steroids	Pollutants	Plant Products
 17β-Estradiol	 DDT	 Genistein (isoflavone)
Pharmaceuticals	 PCB	
 Diethylstilbestrol	 Bisphenol A	 Luteolin (flavone)
 Ethynyl Estradiol	 Nonylphenol	
Fungal Products	 Kepone	 Resveratrol (stilbene)
 Zearalenone	 Lindane	 Coumestrol (coumarin)
	 Phthalate	

TABLE.2 In vitro effects of estrogenic compounds on fetal rat testis or testicular cells.

Protocol	**Age**	**Compound**	**Effects**	**References**
Organ culture	13.5 dpc 14.5 dpc 20.5 dpc	E2 DES E2 DES	↓ number of gonocytes ↓ number of sertoli ↓ number of leydig ↓ testosterone secretion No modification of number of gonocytes	Lassurguere et al.,2003 Delbès et al., 2006
Leydig cell culture	16.5 dpc 20.5 dpc	E2 DES	↓ testosterone secretion	Delbès et al., 2006
Purified gonocyte culture	3 dpp	E2	↑ gonocyte proliferation	Li et al., 1997
Leydig cell culture		Lindane	↓ testosterone secretion ↓ StAR	Ronco et al., 2001 Walsh et al., 2000
Myoid cell culture		Lindane	Membrane depolarization	Silvestroni et al., 1999
Sertoli cell culture		Phthalate	↑ activity of mitochondrial enzyme	Heindel et al., 1992
Organ culture Rat Testis	13 dpc 18 dpc	Phthalate	↓ number of sertoli	Hui et al.,2003

TABLE.3 Effects of in vivo treatment with estrogenic compounds during fetal on testicular development in rodents.

Protocol	Compound	Treatment age	Observation age	Effects	References
Gavage (rat)	Ethinyl estradiol	11-17 dpc	18 dpc	Ootestis, cryptorchidism ↑ number gonocyte ↓ number of Sertoli Leydig cell hyperplasia	Yasuda et al., 1985
Subcutaneous injection (mice)	ZEA DES	9-10 dpc	12-18	Change in gonocyte differentiation Leydig cell hyperplasia	Perez-Martin et al., 1996
Gavage (rat)	Lindane DEA	9-16 dpc	60 dpp	↓ number spermatids	Traina et al., 2003
Gavage (rat)	DES Bpa Genistein	14-21 dpc	0-3 dpp	↑ hsp90 levels in gonocytes ↑ PDGFR	Tuillier et al., 2003 Wang et al., 2004
Subcutaneous injection (rat)	DES	11 and 15 dpc	17	↓ SF-1 mRNA testis ↓ SF-1 protein sertoli	Saunders et al., 1997

Summary of results and overall discussion

Some of the most relevant results reparted in the present thesis are that in the mouse embryonic gonads besides gonadal somatic cells, primordial germ cells (PGCs) the precursors of adult gametes, express estrogen receptor (ER) α and that 17-β-estradiol (E2) via such receptor is able to modulate molecular signalling known to be crucial for their development. Specifically, we demonstrated that PGCs from 11.5-12.5 days post coitum (dpc) mouse embryos express ERα transcripts and protein and that at concentrations of 1-10 nM E2 stimulates rapid (within 20 min) about 3-fold AKT (Ser473) and 2-fold ERK1/2 (Thr202/Tyr204) and SRC (Tyr418) phosphorylation. In addition, the E2 stimulatory effects were associated with increased phosphorylation of the tyrosine kinase KIT (Tyr568/570). While the ER antagonist ICI 182780 was able to abolish all these E2 effects, AKT phosphorylation was inhibited by the PI3K inhibitor LY 294002 and the SRC family inhibitor (PP2). This latter beside SRC phosphorylation was also able to abolish the increased phosphorylation of KIT and ERKs caused by E2. Taken together these results suggest that E2 may modulate via ERα non genomic signalling/phosphorylation cascade in mouse PGCs. This was also supported by the finding that PGCs express MNAR (Modulator of Non genomic Action of estrogen Receptor), a scaffold protein that regulate ER activation in other cell types in which non genomic estrogen action has been demonstrated. Finally, we found that culturing of PGCs in the presence of 10 nM E2 resulted in significant ERα-dependent increase of their number. The results presented in this thesis, provides evidence for novel direct non genomic actions of estrogens on PGCs and suggest that these cells can represent putative target for estrogens and estrogenic compounds during early stages of embryo development in mammals.

In order to study the effect of a prototype of xenoestrogens the pesticide lindane (γ-HCH), an ED, on PGC development in the mouse embryo, we exposed by gavage pregnant mice to 15-30 mg/Kg/bw lindane during the period of PGC migration and gonad colonization (from 8.5 to 11.5 days post coitum, dpc). This treatment resulted in a significant reduction of the number of germ cells within 12.5 dpc testis and ovaries (a maximum of about 25% and 40%, respectively). Similarly, lindane caused a dose-dependent decrease of the PGC number in an in

vitro culture model. Further experiments showed that in such model, lindane induced features of apoptotic cell death in PGCs such as increase in caspase-3 activity, PARP cleavage and TUNEL positivity. A marked increase of the number of PGCs positive for TUNEL staining was also observed in 12.5 dpc gonads of embryos from pregnant mice subjected one day before to acute lindane treatment (60 mg/Kg/bw). Finally, we show that a brief incubation of isolated PGCs with 10^{-5} M lindane resulted in a marked decrease of the basal and KL-induced phosphorylation level of the AKT kinase, known to be crucial for PGC survival. Taken together these results demonstrated that embryo exposure to lindane during early stages of gametogenesis can severely impair the number of germ cells in the foetal gonads; the compound appears to affect PGC survival through a direct pro-apoptotic action likely resulting from its adverse effect on AKT activity in such cells.

The lack of a functional assay able to detect the estogenic activity of estrogens and EDs on somatic cells of the embryonal testis know to express ERα, prompted us to devise a protocol for the expansion of the testis somatic cells expressing estrogen receptor α (ERα) from 12.5 dpc embryos and for their transfection with a plasmid that contains the classical estrogen responsive element (ERE) or the alternative estrogen AP-1 responsive element upstream of the luciferase reporter gene (ERE-Luc and AP1-Luc). StAR immunopositivity of the most part of the $ERα^{+}$ cells grown in culture, allowed their identification as putative Leydig cells. Using the luciferase assay, we evaluated the estrogen activity of 17-ß estradiol (E2), the natural ligand of ERα, on such cells. For comparison, the same assay was carried out on a MCF-7 human breast cancer cell line expressing ERα. The results showed that 24 hr incubation in the presence of E2 resulted in a dose-dependent increase of ERE-Luc activity. At 10^{-8} M E2 concentration ERE-Luc activity increased from 1.7 to 3-fold in the putative Leydig cells and 2.3 to 5.7-fold in MCF-7 cells. These effects were abolished when 10^{-5} M ICI 182.80, an inhibitor of E2 binding, was present in the assay. AP-1-Luc activity was less sensitive to E2 stimulation in both cell types (10^{-8} M E2: putative Leydig cells= 1.2 to 2.7-fold, MCF-7= 3-fold) and the effect of E2 was not abolished by ICI 182.80. Eventually to validate the assay with a xenoestrogen compound, we stimulated the transfected putative Leydig cells and MCF-7 cells with lindane (γ-HCH). Taken together the reported results represent the first evidence of a functional ERα pathway in putative Leydig cells

from early stage of testis development and describe an in vitro assay that can be used to evaluate estrogenic activity of compounds on mammalian embryonic testis.

In conclusion the present data report evidence for the existence of functional estrogen-dependent pathways in embryonic mouse gonads in particular in testis, both germ and somatic cells. The findings that E2 is able to activate via ERα multiple intracellular signalling in PGCs and that the xenoestrogens, lindane negatively interfere with one of these pathways, namely AKT activation are specifically relevant to support the notion of the TDS origin during early stages of testis development. While data are accumulating showing direct effect of estrogens and EDs on gene expression and specific functions of somatic cells of the embryonic testes, in particular Leydig cells, such results on germ cells are lacking and further studies are needed to investigate the effects of these compounds on embryonic germ cell function including epigenetic regulation.

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Publications and additional results

Paper I Rapid estrogen signalling in mouse primordial germ cells (in preparation)

Paper II Pro-apoptotic effects of lindane on mouse primordial germ cells (accepted for publication on Toxicological Sciences)

Paper III Estrogenic assay on putative Leydig cells from mouse embryonic testes (in preparation)

Additional Results Effect of 17-ß-estradiol and lindane on the expression of cell cycle genes of somatic cells of foetal testis

Additional Paper Genistein is an Efficient Estrogen in the Whole-Body throughout Mouse Development

Rapid estrogen signalling in mouse primordial germ cells

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ABSTRACT

In the present work, we report that in the mouse embryonic gonads besides gonadal somatic cells, primordial germ cells (PGCs) the precursors of adult gametes, express estrogen receptor α (ERα) and that via such receptor 17-β-estradiol (E2) is able to modulate molecular signalling known to be crucial for their development. Specifically, we demonstrated that PGCs from 11.5-12.5 days post coitum (dpc) mouse embryos express ERα transcripts and protein and that at concentrations of 1-10 nM E2 stimulates rapid (within 20 min) about 3-fold AKT (Ser473) and 2-fold ERK1/2 (Thr202/Tyr204) and SRC (Tyr418) phosphorylation. In addition, the E2 stimulatory effects were associated with increased phosphorylation of the tyrosine kinase KIT receptor (Tyr568/570). While the ER antagonist ICI 182780 was able to abolish all these E2 effects, AKT phosphorylation was inhibited by the PI3K inhibitor LY294002 and the SRC family inhibitor (PP2). This latter, besides SRC phosphorylation, was also able to abolish the increased phosphorylation of KIT and ERKs caused by E2. Taken together these results suggest that E2 may modulate via ERα non genomic signalling/phosphorylation cascade in mouse PGCs. This was also supported by the finding that PGCs express MNAR (Modulator of Non genomic Action of estrogen Receptor), a scaffold protein that regulate ER activation in other cell types in which non genomic estrogen action has been demonstrated. Finally, we found that culturing of PGCs in the presence of 10 nM E2 resulted in significant ICI-dependent increase of their number. The present study provides evidence for novel direct non genomic actions of estrogens on PGCs and suggest that these cells can represent putative target for estrogens and estrogenic compounds during early stages of embryo development in mammals.

Keywords: PGCs, estrogen, non-genomic effects.

INTRODUCTION

The expression of estrogen receptors α or β (ERα, ERβ) in female and male germ cells has been reported in several species including humans (Kuiper et al., 1996; Saunders et al., 2001; Greco et al., 1992; Jefferson eta l., 2000). In some cases, it has been also demonstrated that the ablation of these receptors or the absence of stimulation by the natural ligand E2 in germ cells (ERKO and ArKO mice) alter some process of their development (Couse and Korach, 1999; Krege et al., 1998; Fisher et al., 1998). The molecular genomic and not genomic bases of such effects remain, however, often to be clarified and the relevance of a direct estrogen effects on germ cells is controversial. While it is likely that this latter may depend on species, sex and the stages of germ cell development, it is undoubted that the expression of ERs renders germ cells a potential direct target of estrogenic compounds widely diffused in the environment. This is particularly important since the increased presence of human made compounds that mimic the action of estrogens termed xenoestrogens in environment and in food and exposure to these compounds during fetal and neonatal period has been hypothized to be the cause of the raise of disorders of male reproductive function, such as the decrease of sperm count, increase in the incidence of testicular cancer and Hypospadias and Cryptorchidism (Testicular Dysgenesis Syndrome-TDS) (Sharpe and Skakkebaek, 1993; Skakkebaek et al. 2001). In this regard, it is to be also considered that even if estrogens or xenoestrogens do not appear to exert effects on processes of germ cell development, their action could result in subtle epigenetic changes of the germ cell genome (Anway MD et al., 2005; Guerrero-Bosagna C et al., 2005) with consequences difficult to foresee for the individual and reproduction of next generation.

For these reasons, it is particularly important to study the expression and functions of ERs in the embryonic precursors of the adult gametes termed primordial germ cells (PGCs) and to verify the presence in such cells of molecular pathways modulated by estrogens.

MATERIALS AND METHODS

PGC isolation and culture

PGCs were obtained from the gonadal ridges (GRs) and gonads of 11.5-12.5 dpc embryos, respectively, of CD-1 mice embryos (Charles River) following the methods described in Pesce et al. (1995) (PGCs purity > 90%).

About 200-300 PGCs were seeded onto a monolayer of STO cells (an embryonic mouse fibroblast cell line purchased from ATCC, USA) in each well of a 96-well plate Falcon tissue culture dish filled with 200 μl of high glucose D-MEM (Gibco), containing non essential amino acids, 0.1 mM 2-mercaptoethanol, 2 mM glutamine, 0.25 mM pyruvate, 75 mg/L penicillin-G, 50 mg/L streptomycin, 0.5 mg/ml N-acetyl-L-cysteine (NAC), and 15% fetal calf serum (Gibco). Where indicated the medium was supplemented with 5 μM forskolin (Sigma) to stimulate PGC proliferation (De Felici et al., 1993). Cultures were carried out in a humidified incubator at 37°C and 5% CO_2 in air (De Felici, 1988). STO cells were propagated in D-MEM plus 10% FCS (see, above). Confluent cell monolayers were inhibited from proliferation by treatment with mitomycin C (Sigma, 10 μg/ml, 3 hr, 37°C). Cells were then harvest by trypsinization and frozen at a concentration of about 5 x 10^6 cells/ml. After thawing, cells were plated at concentration of 2-2.5 x 10^4 cells/ml in 96-well Falcon tissue culture dish. PGCs were identified by alkaline phosphatase (APase) staining (De Felici, 1998) and the number of PGCs was scored 24h after.

RT-PCR analyses

Total RNA was extracted from purified 11.5-12.5 dpc PGCs and somatic cells with RNeasy microkit (Qiagen) in accordance to the manufacturer's instructions. First-strand cDNA synthesis was performed as follows: 200 ng total RNA was reverse transcribed by 50 U of Superscript™II (Invitrogen) using 50 ng random hexamers, in the presence of 0.5 μM deoxynucleotide triphosphates in a final volume of 20 ul. DNA contamination or PCR carry over controls were

performed omitting reverse transcriptase during reverse transcription. The reaction mixture was incubated for 1 h at 42°C, then heat denatured for 15 min at 75 C. 3 ul of the obtained cDNA was used to amplify ERα, ERβ, GRP30 and MNAR.

Primer sequences were:

ERα: 5'-ACCATTGACAAGAACCGGAG-3'

5'-ATAGATCATGGGCGGTTCAG-3'.

ERβ: 5'-TCTGCATAGAGAAGCGATGA-3'

5'-GGCATTCTACAGTCCTGCTG-3'

GPR30: 5'- AGGACTCTGCTCCCCTTAAGCT-3'

5'-GGGCACCCAGAGTGTGTGA-3'

MNAR 5'-CTCGGTTTGAAGGCCTGTGTC-3'

5'-CAAGGAAGTAAGAAGCCCAGG-3'.

Reactions were performed using the following reagents: 0.5uM of primers, Taq polymerase (2U/tube) in a finale volume of 20ul. The amplification consisted of: 35 cycles at 95°C for 1min, 58°C for 1min, 72°C for 1min for ERα ; 35 cycles 95°C for 1min, 62°C for 1min, 72°C for 2 min for ERβ; 35 cycles at 95°C for 1 min, 59°C for1 min, 72°C for 1 min for GPR30; 34 cycles at 95°C for 1min, 62°C for 1min, 72° for 2 min for MNAR. Adult uterus and ovary served as positive controls for ER-α and ER-β respectively and brain for GPR30.

Western blot analyses

Purified PGCs obtained from 30 GRs for each experimental point were resuspended in the culture medium without FCS. After the indicated treatment, 5x SDS sample buffer was added and sample boiled for 5 min. Proteins were separated on 10% SDS-PAGE gels and transferred to PVDF Transfer Membrane Hybond TM-P (Amersham). Membrane were saturated with 5% nonfat dry milk in TBS containing 0,1% Tween 20, for 1 h at room temperature and incubated o/n at 4°C with

the following primary antibody: mouse α-tubulin (1:1000, Sigma), rabbit α-pAKT (pS473) (1:1000, Biosource), rabbit α-pERK1/2 (pTpY202/204) (1:1000, Cell Signaling), rabbit α-pSRC (pY418) (1:1000, Biosource), rat α-ERα (1:500, gently gift by Prof. Green), rabbit α-pKIT (pYpY 568/570) (1:1000, Biosource). Secondary α-mouse, α-rabbit or α-rat IgGs conjugated to horseradish peroxidase (Amersham) were incubated with the membranes for 1 h at room temperature, at 1:10000 dilution in TBS/Tween 20.

Immunostained bands were detected by chemiluminescent method (Amersham) and densitometric analysis of the bands performed using Image Quant software densitometric software (Molecular Dinamics, Sunnyvale Calif.).

Immunolocalization of ERα and OCT4 in gonadal ridges

ERα was immunolocalized in sections of GRs and embryonic gonads following standard immunohystochemistry methods. Briefly, gonads were fixed for 24 h in 4% formalin and then included in paraffin. After deparaffination in xylene, rehydratation in ethanol and rinsing in tap water, the sections were subject to antigen retrieval in 0.01M citrate buffer (pH 6.0) by microwaving for 5 min at full power (700 W) and for 20 min at 60% effect and finally cooled under running tap water. The endogenous peroxidise was blocked by incubating the sections in 3% H_2O_2 in deionised water for 30 min at the dark. ERα monoclonal antibody clone 1D5 (Dako, Glostrup, Denmark; code M7047) was used at 1:50 dilution and slides incubated o/n at 4°C. As negative control, sections were incubated in parallel with only buffer. The incubation was termined by rinsing section with PBS for 5 min. Thereafter, peroxidase-conjugated streptavidin (LSAB+ kit) was added for 1h at RT. After rinsing peroxidise was detected by applying 3.3-diaminobenzidine chromogen solution (DAB) for 5 min. All reagents used for visualization were obtained from DAKP A/S (Glostrup, Denmark).

Anti-OCT4 mouse monoclonal antibody (Santa Cruz, Biotechnology) was used to identify PGCs in adjacent sections. Sections were fixed as previously described, paraffin embedded, antigen retrieved and blocked for non specific signals with PBS-3% BSA for 1h. Sections were incubated o/n at 4°C in the presence of the primary anti-OCT4 antibody (1:250), then washed and incubated for 1h at RT with Alexafluor488 goat anti-mouse (1:500, Molecular Probe).

ERα was also immunolocalized in isolated PGCs. In this case, cells were obtained from gonadal ridges of 12.5 dpc following the EDTA-puncturing method (De Felici and MacLaren, 1983). After attachment to a poly-L-lysine-coated slides PGCs were treated for immunocytochemistry following the protocol described in Norfleet et al. (1999)

Statistical analysis

All experiments were replicated at least three times. The means were tested for homogeneity of variance and analyzed by ANOVA. The level of significance was set at $P \leq 0.05\%$ and $P \leq 0.01\%$.

RESULTS

Expression of ERα in PGCs and gonadal somatic cells

RT-PCR analyses carried out on purified PGCs and somatic cells obtained from 11.5 and 12.5 dpc gonadal ridges showed that both cell types expressed ERα transcripts (Fig.1A). No expression of ERβ or GPR30, a novel G-protein coupled estrogen receptor (Revankar CM et al., 2005), in either cell types was observed. Western blot conducted on the same cell populations revealed also the presence of ERα protein (Fig.1B).

Immunolocalization of ERα in embryonic gonads

We next localized ERα in PGCs both in tissue sections and cells isolated from embryonal gonad. In both 12.5 dpc ovaries and testes, ERα positivity was detected in most of PGCs as revealed from the observation of adjacent section stained for ERα and OCT4-positive cells (Fig.2A). As expected, also some scattered gonadal somatic cells showed ERα positivity that appeared generally stronger than that of PGCs. At higher magnification, in PGCs ERα immunopositivity appeared mainly distributed at the para-plasmamembrane/cytoplasmic region whereas in somatic cells it was mainly in the nucleus (Fig 2B). Similar results were obtained in 11.5 dpc GRs (not shown). Observations on isolated PGCs confirmed these results (Fig. 2C).

17-β-Estradiol enhances AKT, ERK1/2 and SRC phosphorylation

It is now widely demonstrated that beside gene expression estrogen binding to ERs can stimulate activation of multiple cytoplasmic signaling pathways, including many triggered by growth factors such as AKT, SRC and MAPKs (Shupnik MA et al., 2004; Levin ER et al., 2003). In order to verify if ERα expressed in PGCs is functional and able to modulate intracellular signaling pathways, we incubated purified PGCs in the presence of its natural ligand, 17-β-estradiol (E2) and subsequently

analyzed the phosphorylation status of AKT, ERK1/2 and SRC kinases by Western blot. As shown in figures 3 and 4 10^{-8}M E2 induced in PGCs a rapid increase of phosphorylation of these kinases in activation sites. The E2-dependent increase of AKT (Ser473) phosphorylation occurred throughout 5 to 20 min whereas that of ERK 1/2 and SRC (Tyr418) was evident at 5 min and 20 min, respectively. These stimulatory effects were abolished when PGCs were pretreated with ICI 182780, a competitive inhibitor of E2 binding (Fig. 3C, D). Using the specific PI3K inhibitor LY294002, we also found that AKT phosphorylation induced by E2 was downstream PI3K while ERK1/2 and SRC phosphorylation were not (Fig. 3E, and data not shown). As expected, this latter was abolished by SRC inhibitor PP2 (Fig.4A, B). In addition, PP2 was able to abolish either AKT and ERK1/2 increased phosphorylation caused by E2 (Fig. 4 C, D) suggesting the SRC kinase are involved in such action.

17-β-estradiol increase KIT phosphorylation

In some cell types, the binding of E2 to ERα causes phosphorylation of tyrosine kinase receptors such as IGF-1 and EGF and consequently the activation of the downstream signaling pathways associated to these receptors (for a review see, Nilsson et al., 2001). While there is no evidence that mouse PGCs express functional IGF-1 and EGF receptors, the expression in such cells of the KL tyrosine kinase receptor Kit and its crucial role for PGC surival/proliferation are well established (Pesce et al., 1993; De Miguel et al., 2002; Farini et al., 2007). For this reason, we asked whether in PGCs a crosstalk between ERα and KIT was possible. The results showed in Fig. 5 demonstrate that E2 was actually able to elicit a strong phosphorylation of KIT in tyrosine 568/570 and that this action required ERα binding since preincubation of cells in ICI 182780 abolished the effect. Comparing the effect of E2 and KL on AKT phosphorylation in Ser473, downstream KIT activation in PGCs (De Miguel et al., 2002; Farini et al., 2007), we found that KL stimulation was about 3-fold higher than E2 and the combined effect of these compound was not additive (Fig. 5 C, D). The

observation that PP2 was able to abolish KIT phosphorylation induced by E2 (Fig. 5E, F) suggested positive SRC involvement also in such process.

Finally, the presence of transcripts for MNAR (Modulator of Non-genomic Action of ER), a scaffold protein that mediates ER interaction with cytoplasmic signalling components and promotes its interaction with members of the SRC family of tyrosine kinase (Rajhans R et al., 2006; Wong et al., 2002), in PGCs (Fig 6) suggests a possible way through which E2/ ERα could recruit and activate SRC in such cells.

17ß-estradiol induces increase of PGC number in culture

Because activation of PI3K/AKT and ERK1/2 pathways by E2 has been shown to be mitogenic in other cell types (Castoria et al., 2001), we analyzed the effect of E2 on the PGC growth in culture. We found that E2 was able to significantly increase of 20-30% the number of 11.5 dpc PGCs when these cells were cultured for 24h onto STO cells in the presence of 10^{-8}M E2. Such increase was significantly lower to that induced by FRSK, a potent mitogen for PGCs (De Felici et al., 1993) and addictive to that of this latter (Fig.7).

DISCUSSION

Several evidences indicate that estrogens and estrogenic compounds can positively or negatively modulate both female and male reproduction. Estrogens actually can regulate the development and maturation of various reproductive tissues and gonads in mammals. A prerequisite for estrogen action is in general the expression of estrogen receptors in the target cells. As reported in the Introduction, estrogen receptors (ERα, ERβ) have been described in germ cells at various stages of development in mouse and other species. At least, in mammals the physiological relevance of direct estrogen effects on germ cell development remains, however, controversial. In the current study, we show the expression of ERα in terms of transcripts and proteins in PGCs and gonadal somatic cells of embryonic mouse gonads. While the expression of ERα in gonadal somatic cells at this stage was shown by us and others in previous papers (Moe-Behrens et al., 2003; Jefferson et al., 2000; Greco et al., 1991), no evidence for the expression of this receptor in PGCs was reported before. Interestingly, Mitsunaga et al. (2004), described that mouse PGCs express estrogen related receptor β (ERRβ) and that loss of such receptor results in reduction of germ cell number in fetal gonads of both sexes. In order to evaluate the functionality of the ERα expressed by PGCs in terms of its ability, when activated, to trigger rapid intracellular signalling pathways in such cells, we next stimulated purified PGCs with E2 and analysed the phosphorylation status of some kinases known to be crucial in a variety of cytoplasmic signals. Actually, various reports have suggested that E2 interacts with ERα and induces besides classical genomic effect in the nucleus, non genomic rapid cytoplasmic signals, such as activation of adenylate cyclase, PLC, PKC, PI3K, AKT or MAPKs (for a review, see Song et al., 2005; Heldring et al., 2007; Moriarty et al., 2008). The present results demonstrate that AKT and the MAPK family members ERK1/2 phosphorylation is elicited in specific activation sites with a rapid kinetics in mouse PGCs through ERα. Thus suggesting that multiple signalling pathways are rapidly stimulated by E2/ERα complex in such cells. ERs, unlike

many membrane growth factor receptors, have no transmembrane domain and is not intrinsically a membrane protein. To produce rapid non genomic effects, ERs are thought to be located in or near the plasma membrane, where they can access the mechanisms of signal generation. Several laboratories have shown that E2 rapid action requires ERα translocation from the cytosol to the membrane. Currently, it is unknown whether ERα membrane translocation and its activation on the cell membrane is a sequential or independent event. The mechanism of ERα membrane translocation is known to involve adaptor protein Shc and caveolin1 (Chambliss et al., 2000; Schlegel et al., 2001). The immunolocalization of ERα at the para-membrane cytoplasmic region in PGCs observed by us is consistent with these notions.

Because ERα has no intrinsic kinase domain and therefore is not capable of phosphorylating other proteins, the signalling molecules must function directly downstream of, and physically associate with, ERα. At the same time, the signalling molecule must transduce the ERα signal to downstream cascades, leading to the rapid activation of AKT and MAPKs. Estrogen rapidly induces the activation of the PI3K–AKT pathway in a number of cell types including endothelial cells (Koga et al., 2004), cardiomyocytes (Patten et al., 2004), rat neuronal cells (Alexaki et al., 2004) and MCF-7 cells (Ahmad et al., 1999) In the presence of estrogen, ERα interacts with the regulatory subunit of PI3K, p85α, thus triggering activation of the catalytic subunit p110 of PI3K, leading to downstream kinase AKT activation (Castoria et al., 2001). PI3K activation appears involved in the AKT phosphorylation increase induced by E2 in PGCs since its inhibitor LY294002 abolishes such action. Besides PI3K, SRC kinases also appear central in transducing ERα signal in PGCs. Actually, SRC tyrosine kinases has been identified as a crucial molecule downstream of ERα by physical interaction with ERα, and might mediate estrogen rapid action (for a review, see Song et al., 2005). Our observations that in PGCs E2 is able to increase the phosphorylation level of SRC in the activation site Tyr 418 and that the specific SRC inhibitor PP2 prevents, in addition of such phosphorylation, also the increased phosphorylation of AKT and ERK1/2 induced by E2 support the fact that SRCs are central in transducing the ERα signal in such cells.

To complicate the picture of E2 action on mouse PGCs, we found that the hormone was also able to increase the phosphorylation of KIT receptor in its autophosphorylation sites tyr 568/570 and that this effect was dependent by the SRC activity since it was inhibited by PP2. In some cell types, it was already known that the binding of E2 to ERα cause phosphorylation of tyrosine kinase receptors as IGF-1 and EGF and consequently their transactivation (for a review, see Nilsson et al., 2001), here we show for the first time that also KIT receptor can be a target for E2. How SRCs cause increased KIT phosphorylation in tyr 568/570 in unclear since the only known KIT phosphorylation site for SRC is tyr 900 (Lennartsson et al., 2003). In any case, the presence of MNAR transcripts in PGCs suggest that as in other cell types in which estrogen non genomic action is mediated by SRCs, this scaffold protein may serve to link ERα to these kinases. Actually, MNAR has been reported to interact directly with both ERα and SRCs in the cytosol, stabilizing the protein complex and leading to SRC activation by unfolding SRC and inducing SRC Y-418 phosphorylation (Barletta et al., 2004).

AKT phosphorylation in Ser473 resulting from activation of KIT by its natural ligand KL (Farini eta l., 2007), was, however, much more efficient (at least 3 times) than that induced by E2 and the action of both compound on this phosphorylation site was not additive. This last results indicates that threshold phosphorylation of AKT on this site is reached at the concentration of 100ng/ml KL used in this assay. It remains to be established whether AKT phosphorylation induced by E2 is due to KIT transactivation or directly to the action of the putative ERα/MNAR/SRC/PI3K complex. On the other hand, it is likely thought that the increased E2-dependent phosphorylation of ERK1/2 is primarily due to such complex since KIT activation in PGCs do not lead to ERK1/2 phosphorylation (Farini et al., 2007).

A summary diagram of the possible signalling pathways of E2 in mouse PGCs is shown in Fig. 8.

Although as reported above the functional relevance of ER expression is often controversial, it is clear, that at least at certain stages of formation, estrogens can directly affect germ cell

development. For example, in early meiotic spermatocytes and elongating spermatids of the human testis low concentrations of E2 (10^{-9} and 10^{-10} mol/L) effectively inhibited male germ cell apoptosis (Pentikainen et al., 2000) and in early postnatal rat gonocytes E2 stimulates proliferation in culture (Li et al., 1997). The finding reported here that E2 is able to increase the number of PGCs in culture suggests that the activated signallings can eventually converge on stimulation of PGC proliferation.

If the E2 effects on PGCs described in the present paper have functional relevance in vivo for the correct PGC development in mammals remains to be established by future studies. In any case ER expression and functionality demonstrated here in mouse PGCs, are indicative of potential targeting of these cells by estrogens and estrogenic compounds. This is relevant because as reported in the Introduction such compounds can also exert subtle epigenetic changes in the genome. Indeed PGCs are not only responsible for the formation of the gametes but are also the cells through which the genome is transmitted to the next generations.

Acknowledgements

The present study was supported by European Community project "GENDISRUPT" (QLK4-CT-2002-02403) and Ministero del Lavoro e della Previdenza Sociale grant n. 1650.

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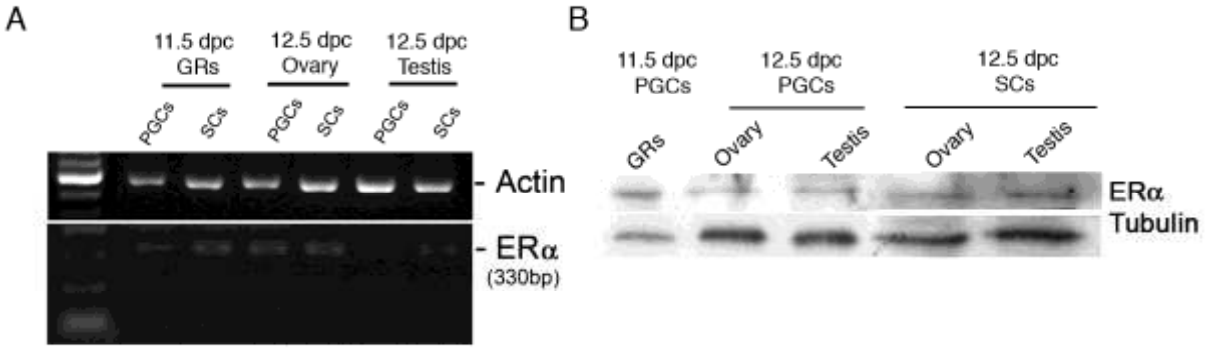

Figure 1. ERα expression in PGCs and gonadal somatic cells (SCs). A RT-PCR analyses of ERα transcripts in PGCs and SCs obtained from 11.5 dpc gonadal ridges (GRs) and 12.5 dpc ovaries and testes. **B** Western blot for ERα protein in the same cell population. For each analysis a representative results of three performed experiments is show.

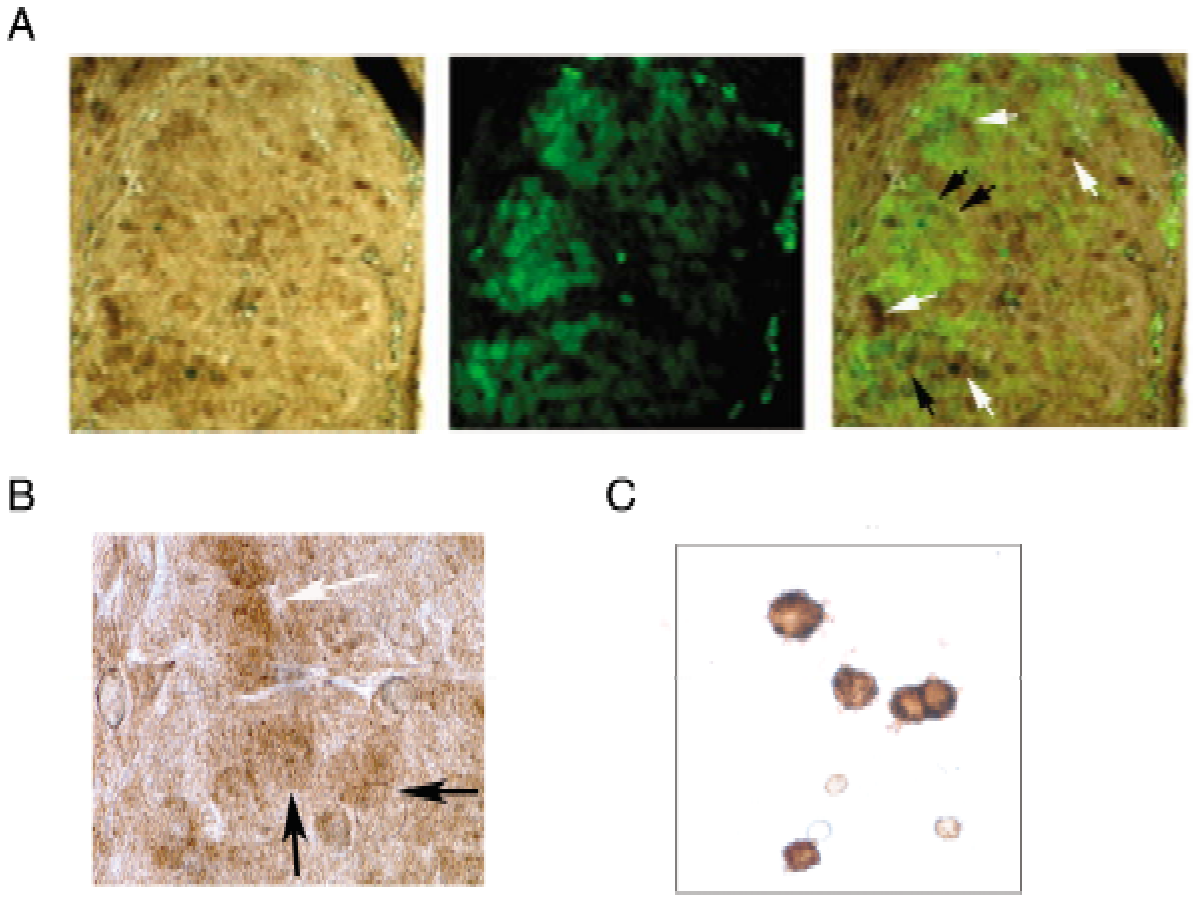

Figure 2. ERα immunohystochemistry in tissue sections and isolated PGCs of embryonic gonads. A From left to right two adjacent sections of a 12.5 dpc testis showing ER-α staining and OCT4-positive PGCs and a merged picture of these sections highlighting $ERα^+$ $OCT4^+$ PGCs (black arrows) and $ERα^+$ $OCT4^-$ SCs (white arrows). Original magnification 40x. **B** A field of a section showing $ERα^+$ PGCs and SCs. Original magnification 100x.**C**. Immunocytochemistry for ERα in purified 12.5 dpc PGCs. Note ERα localization at the para-plasmamembrane cytoplasmic region.

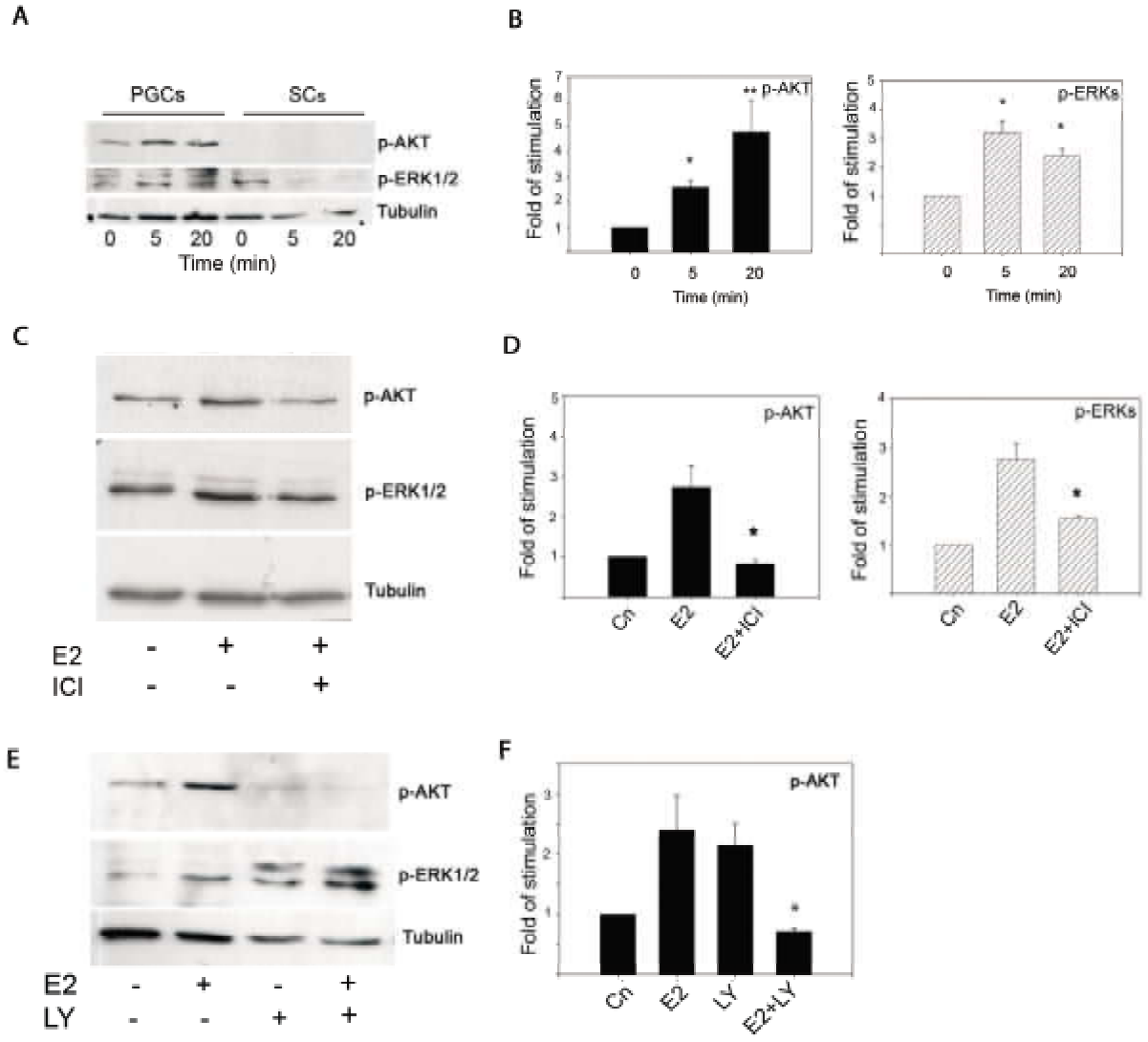

Figure 3. Western blot analyses of the effect of E2 on AKT and ERK1/2 phosphorylation. A PGCs from 12.5 dpc gonads (left panel) were incubated in presence of 10^{-8}M E2 for the indicated times. Somatic cells (SCs) (right panel) were treated in the same way. **C** PGCs from 12.5 dpc gonads were preincubated for 10 min with or without 10^{-5}M ICI 182780, before 10^{-8}M E2 stimulation for 20 min. **E** PGCs were preincubated for 30 min with or without LY294002 (5 µM) before stimulation with 10^{-8}M E2 for 20 min. Cell lysates were analysed for p-AKT (ser 473) and for p-ERK1/2 (Thre202/Tyr204). The membranes were reprobed with mouse anti-tubulin. All experiments were performed at least 3 times and typical blots are shown. **B, D and F** Densitometric analyses of all Western blots performed. Results of three experiments were normalized to tubulin and plotted (mean ± SEM) in relation to control (C=1). *, $p<0.05$, **, $p<0.01$.

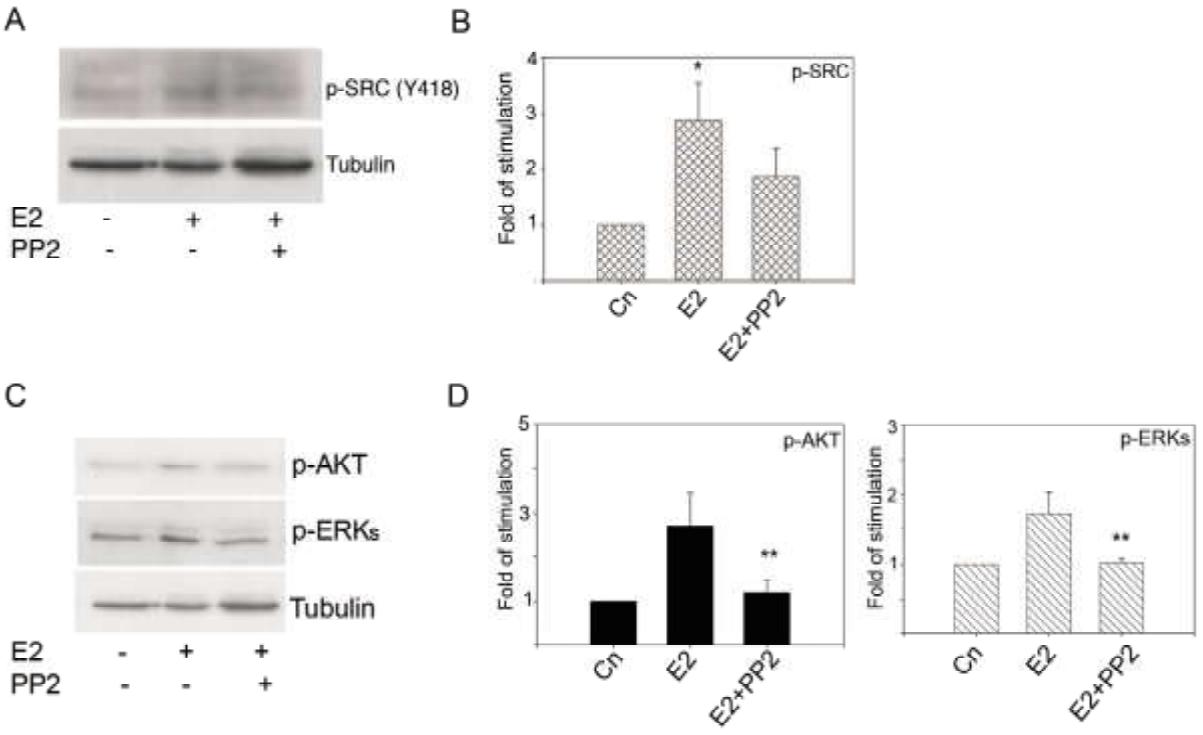

Figure 4. Western blot analyses of the effect of E2 on SRC phosphorylation and of PP2 on SRC, AKT ERK1/2 phosphorylation. A PGCs from 12.5 dpc gonads were incubated in presence of 10^{-8}M E2 for 20 min. Cell lysates were analysed for p-SRC and the same membrane was reprobed with mouse anti-tubulin. **C** 12.5 dpc PGCs were preincubated for 30 min with or without PP2 (20ng/ml) before stimulation for 20 min with 10^{-8}M E2. Cell lysates were blotted and incubated with p-AKT, p-ERK1/2 and p-SRC. The same membrane was reprobed with mouse anti-tubulin. All experiments were performed at least 3 times and typical blot of the tree experiment is show. **B** and **D**. Densitometric analyses of the Western blot results. Results were normalized to tubulin in each sample and plotted (mean ± SEM) in relation to control (C=1). *, $p<0.05$, **, $p<0.01$.

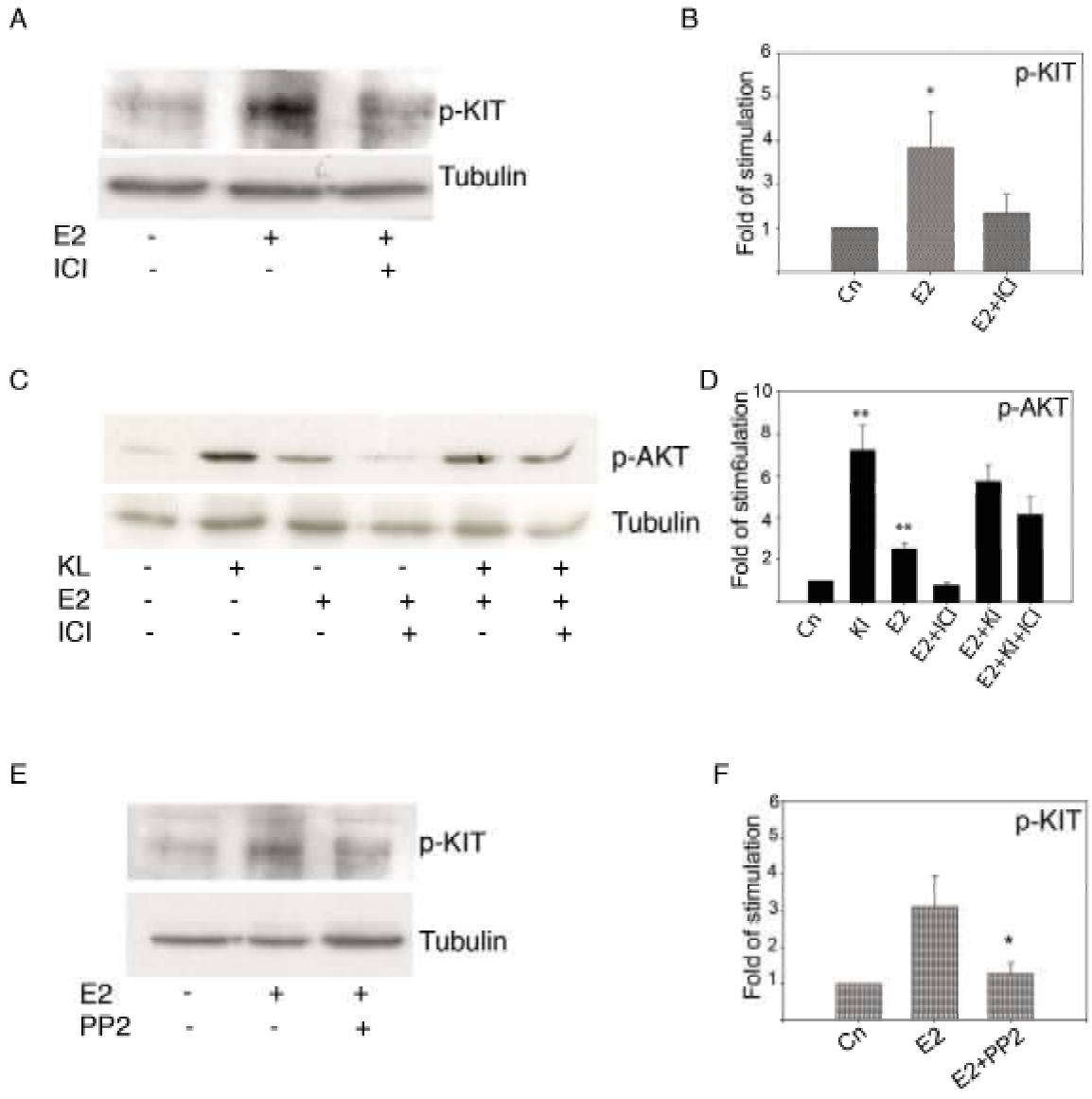

Figure 5. Western blot analyses of the effect of E2 on Kit phosphorylation. A 12.5 dpc PGCs were preincubated for 10 min with or without 10^{-5}M ICI 182780, before 10^{-8}M E2 stimulation for 20 min. Cell lysates were analysed for p-KIT and the same membrane was reprobed with mouse anti-tubulin. **C** Comparison of Kit phosphorylation induced by E2 or KL. PGCs were stimulated with 10^{-8}M E2 for 20 min or KL (100ng/ml) for 10 min or preincubated for 10 min with E2 and then stimulated for the last 10 min with KL. ICI 182780 (10^{-5} M) was added 10 min before hormonal stimulations and together with E2 and KL. Cell lysates were blotted and incubated with anti-p-AKT and reprobed with anti-tubulin for loading control. All experiments were performed at least 3 times and typical blots are shown. **E** 12.5 dpc PGCs were preincubated for 30 min with or without PP2 (20ng/ml) before stimulation for 20 min with 10^{-8}M E2. Cell lysates were blotted and incubated with p-KIT. **B D** and **F**. Densitometric analysis of the all Western blots performed. Results were normalized to tubulin in each sample and plotted (mean ± SEM) in relation to control (C=1). *, $p<0.05$, **, $p<0.01$.

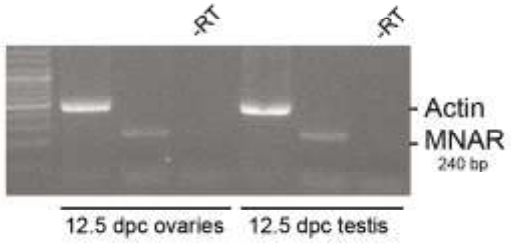

Figure 6. PGCs express MNAR transcripts. RT-PCR analyses of MNAR in purified 12.5 dpc PGCs. Actin was used as control housekeeping gene.

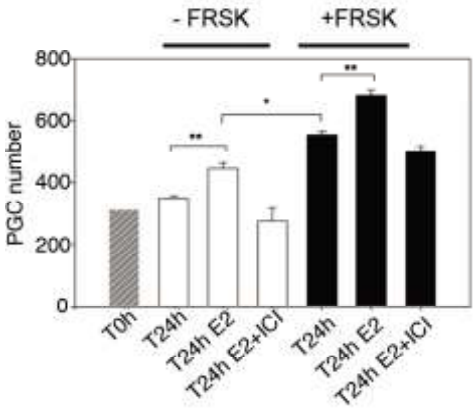

Figure 7. Effect of 17ß-estradiol on the number of PGCs in culture. A 11.5 dpc PGCs were cultured for 24 h onto STO cell monolayers in the presence of 10^{-8}M E2. ICI 182780 (10^{-5}M) was added 1 h before E2 and maintained throughout the culture period. Results are given as the mean ± SEM of three independent experiments. ** = $p<0.01\%$; * = $p<0.05\%$.

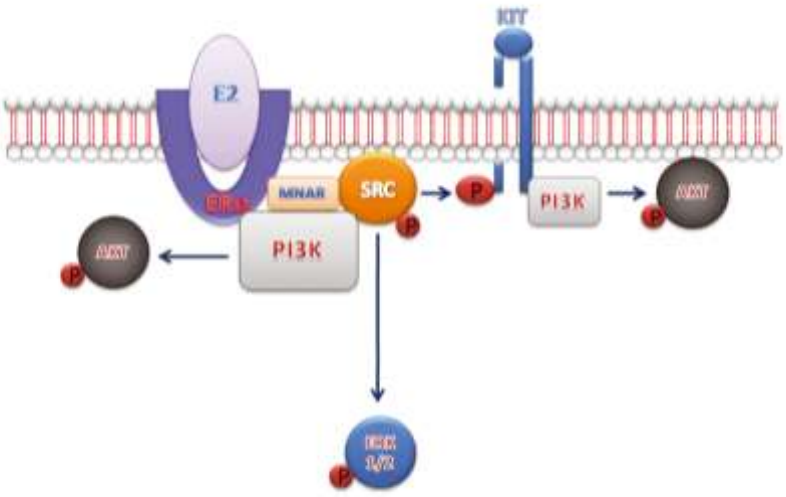

Figure 8. Proposed model for signal transduction pathways activated by E2 in mouse PGCs. Binding of E2 to ERα allows the formation of a complex with MNAR that binds SRCs and leads to its activation; ERα and SRC bind PI3K that after activation mediates AKT phosphorylation; activated SRC is also able to trigger the activation pathway of ERK1/2. Eventually, SRC is responsible to cross-phosphorylation of KIT that in turn leads to the activation of the PI3K/AKT pathway.

OXFORD UNIVERSITY PRESS Toxicological Sciences

PRO-APOPTOTIC EFFECTS OF LINDANE ON MOUSE PRIMORDIAL GERM CELLS

Journal:	*Toxicological Sciences*
Manuscript ID:	draft
Manuscript Type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	LA SALA, GINA; TOR VERGATA, DEP.PUBLIC HEALTH AND CELL BIOLOGY
Key Words:	apoptosis < Carcinogenesis, endocrine disruptors < Endocrine Toxicology, kinase regulation < Gene Expression/Regulation, embryo < Reproductive & Developmental Toxicology, pesticides < Agents

Pro-apoptotic effects of lindane on mouse primordial germ cells

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ABSTRACT

Lindane (γ-HCH) was examined for its effect on primordial germ cell (PGC) development in the mouse embryo. We found that exposure by gavage of pregnant mice to 15-30 mg/Kg/bw lindane during the period of PGC migration and gonad colonization (from 8.5 to 11.5 days post coitum, dpc) resulted in a significant reduction of the number of germ cells within 12.5 dpc testis and ovaries (a maximum of about 25% and 40%, respectively). Similarly, lindane caused a dose-dependent decrease of the PGC number in an in vitro culture model. Further experiments showed that in such model, lindane induced features of apoptotic cell death in PGCs such as increase in caspase-3 activity, PARP cleavage and TUNEL positivity. A marked increase of the number of PGCs positive for TUNEL staining was also observed in 12.5 dpc gonads of embryos from pregnant mice subjected one day before to acute lindane treatment (60 mg/Kg/bw). Finally, we show that a brief incubation of isolated PGCs with 10^{-5} M lindane resulted in a marked decrease of the basal and KL-induced phosphorylation level of the AKT kinase, known to be crucial for PGC survival. Taken together these results demonstrate that embryo exposure to lindane during early stages of gametogenesis can severely impair the number of germ cells in the foetal gonads; the compound appears to affect PGC survival through a direct pro-apoptotic action likely resulting from its adverse effect on AKT activity in such cells.

Keywords: PGCs, lindane, γ-HCH, apoptosis, Akt

INTRODUCTION

Lindane, the γ-isomer of hexachlorocyclohexane (γ-HCH), is one of the oldest synthetic pesticides still in use worldwide. Lindane has been used extensively to control malaria, but is more commonly used to eradicate insects in agriculture and to treat lice infestation in humans, poultry, and livestock. Because of its widespread use and chemical stability, this pesticide has become widely distributed in ecosystems and is now a global pollutant considered. Lindane may act as endocrine disrupter (ED) through inhibition of steroid hormone synthesis or other not well characterized mechanisms including action on estrogen receptors (ERs) (Maranghi et al., 2007; Tezak et al., 1992). Numerous reports have shown that lindane adversely affects reproductive function in animals (for references, see Walsh et al., 2000). For example, in adult male rats, chronic exposure to lindane markedly reduces serum testosterone levels, epididymal sperm counts, and sperm motility, whereas in guinea pigs, it damages seminiferous tubules and completely arrests spermatogenesis (Chowdhury et al., 1994; Chowdhury et al., 1990; Chowdhury et al., 1987; Dikshith et al., 1978; Prasad et al., 1995). Moreover, long-lasting effects of lindane on mouse spermatogenesis following in utero exposure have been reported (Dalsenter et al., 1997; Traina et al., 2003). In adult female mice, rabbits and rats, lindane reduces serum estrogen and progesterone levels, whereas in pregnant mice and minks, it decreases whelping rate and litter size (Beard et al., 1985; Chadwick et al., 1998; Sircar et al., 1990; Srivastava et al., 1993; Sircar et al., 1989). In an in vitro model, the pesticide abolishes oocyte directed follicle organizing activity (Li et al., 1997). Exposure to lindane during the first four days of pregnancy completely prevents implantation in mice, but normal pregnancy results when estrogen and progesterone were coadministered with the compound (Scascitelli et al., 2003). As far as we know, no works have addressed the effect of lindane on early gametogenesis. In the present

paper, we carried out in vitro and in vivo experiments in mouse aimed to investigate possible effects of the pesticide on the development of primordial germ cells (PGCs) from which the gametes of adult originate.

MATERIALS AND METHODS

Animals and treatments

Experiments were carried out in compliance with the ethical provisions enforced by the European Union and authorized by the National Committee of the Italian Ministry of Health on in vivo experiments.

CD1 female mice (Charles River, Italy) were mated with CD1 male mice and the detection of a vaginal plug the morning following mating was designated 0.5 day post coitum (dpc). Pregnant females were randomly divided into control groups (vehicle) and γ-HCH treated groups (15, 30 and 60 mg/kg bw) (Sigma-Aldrich) suspended in olive oil (Sigma-Aldrich). Treatments were administrated by gavage as indicated. Dose level was selected according to the literature and in order to avoid evident signs of maternal toxicity or embryotoxicity (Traina et al., 2003). Mice were kept under standard laboratory conditions (22±0.5°C room temperature), 50-60% relative humidity, 12 h dark-light alternation with 12-14 air changes/h) with water and food (NIH-07 Open Formula diet purchased from Mucedola, Bergamo, Italy) available *ad libitum*. Ovaries and testes were dissected from embryos obtained from 12.5 dpc pregnant females. One gonad of each pair was fixed in 70% ethanol and stored at 4°C, the controlateral was incubated in trypsin-EDTA calcium-free PBS for 5 min for complete tissue disaggregation. Ethanol fixed gonads were embedded in paraffin, serial sectioned and PGCs scored as reported in (Iona et al., 2002). Three 5µl samples of the disaggregated cell suspension obtained from the controlateral gonads were transferred to a poly-L-lysine-coated slide, fixed with 4% paraformaldeyde and stained for APase activity (De Felici, 1998). APase positive PGCs were counted in every sample under a standard light microscope. Where indicated sections of paraffin embedded gonads of 12.5 dpc embryos from control and treated

pregnant mice were processed for TUNEL histochemistry (Roche, In Situ Cell death Detection Kit, Fluorescein, cat. N. 11684795910) according to the manufacturer's instructions.

PGC isolation and culture

PGCs were obtained from the gonadal ridges of 11.5-12.5 dpc CD-1 mice embryos (Charles River, Italy) following the methods described in Pesce et al., 1995 (PGCs purity > 90%). About 200-300 PGCs in 200µl of culture medium were seeded in each well of a 96-well Falcon dish onto monolayers of STO cells (an embryonic mouse fibroblast cell line purchased from ATCC, USA). The culture medium consisted of high glucose D-MEM (Gibco), containing non essential amino acids, 0.1 mM 2-mercaptoethanol, 2 mM glutamine, 0.25 mM pyruvate, 75 mg/L penicillin-G, 50 mg/L streptomycin, 0.5 mg/ml N-acetyl-L-cysteine (NAC), 5 µM forskolin purchased from Sigma and 15% fetal calf serum (Gibco). Cultures were carried out in a humidified incubator at 37°C and 5% CO_2 in air. STO cells were propagated in D-MEM plus 10% FCS (see, above). Confluent cell monolayers were inhibited from proliferation by treatment with mitomycin C (Sigma, 10 µg/ml, 3 hr, 37°C). Cells were then harvest by trypsinization and frozen at a concentration of about 5 x 10^6 cells/ml. After thawing, cells were plated at concentration of 2-2.5 x 10^4 cells/ml in 96-well Falcon tissue culture dish.

APase, TUNEL and BrdU staining in cultured PGCs

The number of apoptotic PGCs in culture was scored by dual staining with APase and TUNEL cytochemistry as previously described (Farini et al., 2005). Briefly cells were washed in D-MEM medium, fixed in 70% ice-cold ethanol (5 min) and stained for APase as reported in Farini et al., 2005. APase and APase/TUNEL-positive cells were scored in random fields as reported above using a light transmitted microscope. The BrdU incorporation assay was performed as described in Farini et al., 2005. APase and APase/BrdU positive cells were scored in random fields as reported above.

Western blotting analysis

Purified PGCs were resuspended in the culture medium without FCS and after the indicated treatment, 5x SDS sample buffer was added to cells and samples boiled for 5 minutes. Proteins were separated on either 10% or 12% SDS-PAGE gels and transferred to PVDF Transfer Membrane Hybond™-P (Amersham). Membranes were saturated with 5% nonfat dry milk in TBS containing 0.1% Tween 20, for 1h at room temperature and incubated o/n at 4°C with the following primary antibody: mouse α–tubulin (1:1000, Sigma), rabbit α-AKT and α-pAKT (Ser473) (1:1000, Biosource), rabbit anti-ERK1/2 and anti-pERK1/2 ($pTpY^{202/204}$) (1:1000, Cell Signalling), rabbit α–PARP (1:1000, Santa Cruz) and rabbit α–caspase-3 (1:1000 Cell signalling). Secondary α-mouse or α-rabbit IgGs conjugated to horseradish peroxidase (Amersham) were incubated with the membranes for 1h at room temperature, at 1:10000 dilution in TBS/Tween20. Immunostained bands were detected by chemiluminescent method (Amersham) and densitometric analysis of the bands performed using Image Quant densitometric software (Molecular Dynamics, Sunnyvale, Calif.)

Statistical analysis

All experiments were replicates at least three times. The means were tested for homogeneity of variance and, unless otherwise indicated, analyzed by ANOVA. The level of significance was set at $P<0.05\%$ and $P<0.01\%$.

RESULTS

Lindane causes a marked reduction of the germ cell number both in vivo and in vitro.

In order to investigate whether embryo exposure to lindane affects early gametogenesis, 15-30 mg/Kg lindane was administered to pregnant mice daily by gavage from 8.5 to 11.5 dpc, the main period of migration and gonadal ridge colonization by the gamete precursors termed primordial germ cells (PGCs) (Tam and Snow, 1981). The number of germ cells in 12.5 dpc gonads was then scored in the whole cell populations obtained from disaggregated gonads and in some cases in gonad tissue sections (see, Material and Methods). The results of the counts performed in disaggregated cells reported in Fig. 1 and Table 1-2 (see, supplementary data), show that lindane treatment caused a dose-dependent decrease of the number of germ cells both in the developing testes and ovaries with a maximum of about 25% and 40%, respectively. Similar results were obtained when counts were performed in tissue sections of the controlateral gonads (data not shown). To evidence a possible direct effect of lindane on PGCs, we next performed in vitro culture experiments in which purified populations of PGCs obtained from 11.5 dpc embryos were incubated in the presence of increasing concentrations of lindane for three hours before culture onto STO cell monolayers. We found that such treatment resulted in a significant dose-dependent decrease of the number of PGCs after 24 hr of culture (Fig. 2).

Lindane induces apoptotic features in PGCs

Acute exposure of 11.5 dpc pregnant mice to 60 mg/Kg/bw lindane, caused a highly significant increase of the number of TUNEL positive PGCs within 12.5 dpc gonads (Fig. 3A e B). No significant increase of the number of TUNEL positive PGCs was, however, observed in tissue sections of gonads dissected from embryos in which high PGC loss was scored as a result of

the daily lindane treatment described above (data not shown), thus suggesting that this last treatment caused PGC loss mainly before their arrival into the gonadal ridges.

Next, we found that in culture, the decrease of PGC number caused by 10^{-5} M lindane was associated to increased number of PGCs positive to TUNEL (Fig. 4A and B) whereas the percentage of the PGCs able to incorporate BrdU did not change (Fig. 4C and D).

These last in vitro culture results suggest that the decrease of PGC number caused by lindane is due to a direct induction apoptosis in such cells. In order to verify such a possibility, we analysed the presence of active caspase-3 and poly-ADP-ribose polymerase (PARP), two well recognized markers of apoptosis, in PGCs treated for three hours with 10^{-5} M lindane. Western blotting analyses showed the presence of cleaved form of both caspase and PARP, indicative of enzyme activation, in PGCs treated with lindane but not in control PGCs (Fig. 5A and B).

Previous studies by us and others, have clearly shown that KL/Kit system and the downstream effector AKT kinase are important players counteracting PGC apoptosis and consequently exerting a positive control on PGC survival (De Miguel et al., 2002; Farini et al., 2007; Pesce et al., 1993). Interestingly, we found that 30 min incubation of PGCs in the presence of 10^{-5} M lindane reduced significantly the basal level of phosphorylation of AKT in Ser473 (Fig. 6A) and abolished the increase of such phosphorylation induced by 100 nM KL (Fig. 6B); the same treatment did not affect AKT phosphorylation in somatic cells from the same gonads (see Fig.1, supplementary data). Finally no effect of lindane treatment on ERK1/2 phosphorylation in PGCs was observed. (Fig.6C)

DISCUSSION

Lindane is considered a reprotoxic compound; the reproductive No Observed Adverse Effect (NOAEL) for lindane is 1.7 mg/kg body weight/day. Indeed recent studies, reviewed in the

Introduction, have shown that in adult animals consumption of lindane can damage male and female reproduction. Long-lasting effects on male reproductive capability could occur in the adult organism also as a consequence of the exposure to lindane during the prenatal period, as suggested by in vivo studies in rats (Dalsenter et al., 1997a; Dalsenter et al., 1997b), minks (Beard et al., 1998), rams (Beard et al.,1999), rabbits (Fausto et al., 2001) and mice (Traina et al., 2003; Maranghi et al., 2007). In the present paper, we show that lindane given to pregnant mice through the digestive system during the early period of germ cell formation causes a marked reduction of the female and male germ cells within the developing gonads. Moreover, we observed that a relatively brief preincubation for three hours of isolated PGCs in lindane impairs their ability to grow in culture. Different mechanisms have been hypothesized to explain the action of lindane on reproductive tissue according to the in vivo or in vitro experimental model being investigated, including interaction with hormone receptors (Danzo et al., 1997; Flouriot et al., 1995; Schrader et al., 2000), impairment of hormone metabolism and transport (Walsh et al., 2000), or inhibition of intercellular communication within reproductive tissues (Criswell et al., 1995a; Criswell et al., 1995b; Tiemann et al., 1999). Although it has been reported that lindane neither binds to estrogen receptors (Soto et al., 1995), nor it interferes with the binding of dihydrotestosterone (DHT) to androgen-binding protein (ABP) (Danzo et al., 1997), Maranghi et al. (Maranghi et al., 2007) showed that ERβ is a potential target for this compound acting as ED during the female reproductive system development. Lindane can also interact directly with gametes. Important physiologic functions, such as the induction of the acrosomal reaction, can be impaired by short-term exposure of spermatozoa to environmentally relevant doses of lindane (Silvestroni et al., 1999). In an in vitro model, lindane abolishes rat oocyte directed follicle organizing activity by inhibiting gap junction communication (Li et al., 1997).

Experiments performed by us to investigate the mechanism of PGC loss in vivo and growth reduction in culture caused by lindane revealed that this compound negatively affects

the survival of PGCs by directly inducing in such cells features typical of the apoptotic cell death including caspase-3 activation, cleavage of PARP and DNA breaks, this last evidenced by TUNEL positivity. These results together with the increased number of TUNEL positive PGCs observed in the foetal gonads following acute exposure of pregnant mice to lindane, strongly suggest that the pesticide exerts direct pro-apoptotic effect on PGCs. This does not exclude, however, that in vivo lindane might also indirectly affect PGC survival, for example by inhibiting gap junction communication between PGCs and somatic cells. Lindane is a general gap junction blocker (Li et al., 1997; for additional references see Loch-Caruso et al., 2003) and PGCs are able to form gap-junctions with the surrounding somatic cells (De Felici et al., 1983; Francis et al., 2006) by which their survival may also depend (Francis et al., 2006).

In a final series of experiments, we identified the likely target of the pro-apoptotic action of lindane on isolated PGCs in the rapid impairment of the phosphorylation status of the AKT. This kinase is downstream the KL/Kit signalling known to be crucial for sustaining PGC survival (De Miguel et al., 2002; Farini et al., 2007; Pesce et al., 1993). Besides the observation that the estrogen receptor antagonist ICI-182780 does not inhibit the effects of lindane on PGCs (data not shown), excluding its action on estrogen receptors, we have not further investigated how lindane can interfere with AKT phosphorylation in PGCs. In this regard, it is important to note that lindane is highly lipophilic and incorporates into biological membranes (Golubev, 1993). Moreover, it decreases the membrane concentration of phosphatidylinositol (4,5)-bisphosphate (PIP_2), the immediate precursor of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) and primary regulator of AKT phosphorylation (Sauviat et al., 2002). Therefore we postulate that also in PGCs AKT phosphorylation is compromised by the diminished availability of PIP_2 elicited by lindane.

Whatever the precise mode of lindane action, PGC loss reported in the present paper as result of the exposure of pregnant mice to this compound could represent a potential risk for gametogenesis and reproduction in mammals.

Acknowledgements

The present study was supported by European Community project "GENDISRUPT" (QLK4-CT-2002-02403) and Ministero del Lavoro e della Previdenza Sociale grant n. 1650.

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FIG.1 Continuous exposure of mouse embryos to lindane from 8.5 to 11.5 dpc causes a decrease of the PGC number in 12.5 dpc gonads. Box plot representation of PGC number in 12.5 dpc male (A) and female (B). The box plot illustrates unusual distributions, sample size in each group and the confidence interval about the median. Each box bounds the first and the third quartiles (referred to as the interquartile range) encompassing 50% of the data and includes the median, upper and lower hinges, as indicated by horizontal lines within the box. Dispersion of the data above and below this range is marked by vertical bars that extend to the most extreme values, including the adjacent values inside the upper and lower fences, and by individual outliers beyond this range (filled circles). The significance of the differences between experimental values was assessed by the Kruscal-Wallis One Way Analysis Test. * = $p<0.05\%$ was considered to be statistically significant.

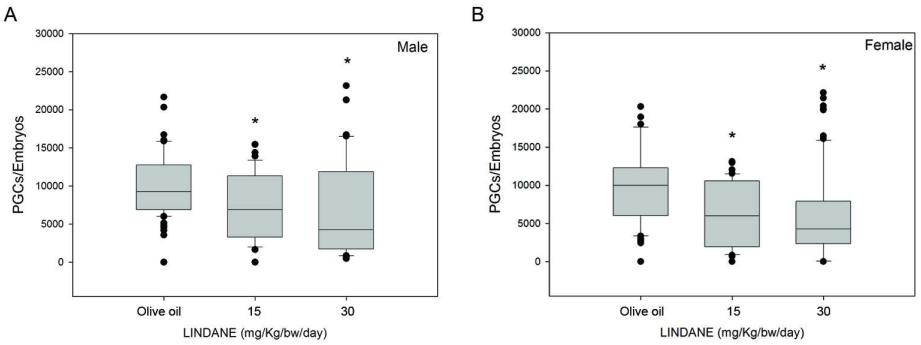
FIG.2 Lindane causes a dose-dependent decrease of the PGC number in culture. 11.5 dpc PGCs were incubated in suspension for 3h in the presence of the indicated concentrations of lindane, washed and cultured for further 24h onto STO cell monolayers without lindane. The number of PGCs identified by APase staining attached to STO cell monolayer in parallel replicates after 3h of culture was normalized to 100%. Results are given as the mean ± SEM at least three independent experiments. ** = $p<0.01\%$; * = $p<0.05\%$.

FIG.3 Acute exposure of 11.5 dpc embryos to 60 mg/Kg bw lindane causes a marked increase of TUNEL positive PGCs in 12.5 dpc of the gonads. A. Representative sections of a 12.5 dpc ovary from CN (control) and lindane treated embryos subjected to the indicated staining (arrow indicate APase$^+$ TUNEL$^+$ PGCs). Original magnification in A=100X; **B.** Quantitative evaluation of the percentage of APase$^+$ TUNEL$^+$ PGCs in 12.5 dpc gonads. The TUNEL$^+$ PGCs were counted from six randomly selected serial sections of four gonads of different embryos. * = $p<0.05\%$.

FIG.4 Effect of lindane on PGC apoptosis and proliferation in culture. 11.5 dpc PGCs were exposed to 10^{-5}M lindane for 3h before culturing for 24h onto STO cell monolayers. TUNEL cytochemistry (A) or BrdU labeling (C) were performed as reported in Material and Methods.. APase$^+$ and Apase$^+$/TUNEL$^+$ or APase$^+$ and APase$^+$/BrdU$^+$ PGCs were scored in at least three random fields as reported above. The results are represented as the mean ± SEM of three independent experiments. * = $p<0.05\%$.

FIG.5 Lindane causes activation of caspase-3 and PARP in PGCs. 12.5 dpc PGCs were incubated for 3h with or without 10^{-5}M lindane at 37°C. Cell lysates were analyzed by Western blot with antibody against the cleaved (activated) caspase-3 (17/19 kDa) (A) or against uncleaved (116 kDa) and cleaved (activated) (85 kDa) PARP (B). The experiments were performed at least three times and typical blots are shown.

FIG.6 Western blot analyses of the effect of lindane on AKT and ERK1/2 phosphorylation in PGCs. 12.5 dpc PGCs were incubated in presence of 10^{-5}M lindane for the indicated times. In the experiment showed in B, before stimulation with 100 ng/ml KL (Immunological Science) for 10 min PGCs were incubated with or without lindane 10^{-5}M for 20 min. Typical blots and densitometric analyses are shown on the left and on the right, respectively. For densitometric analyses results were normalized to total AKT and plotted (mean ± SEM) in relation to control made = 1. ** = $p<0.01\%$; * = $p<0.05\%$.



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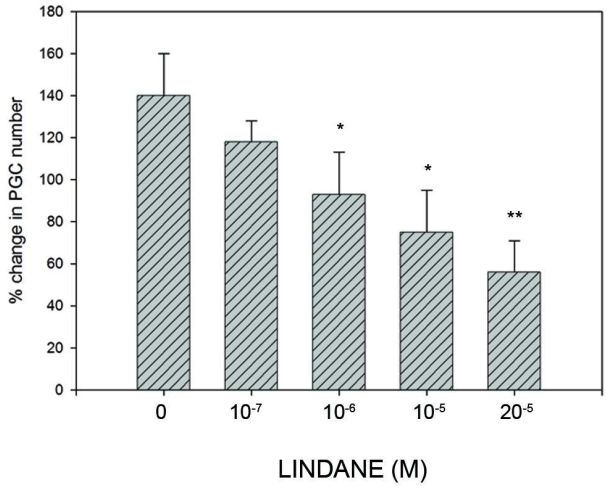

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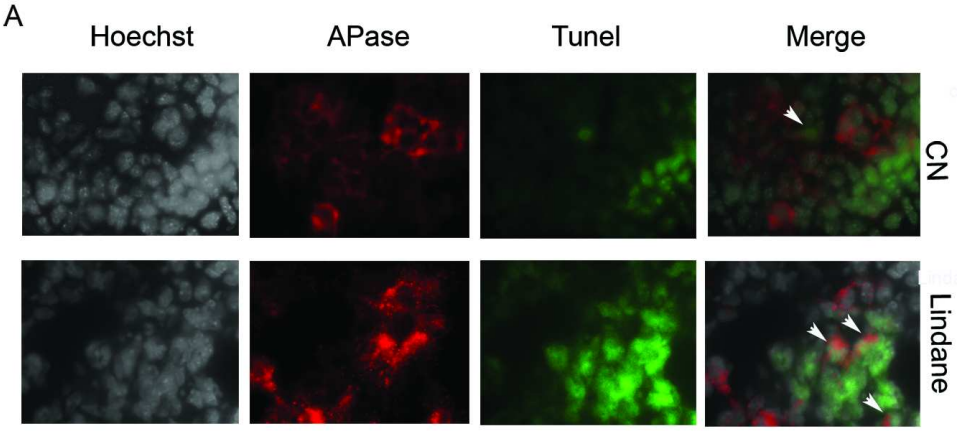

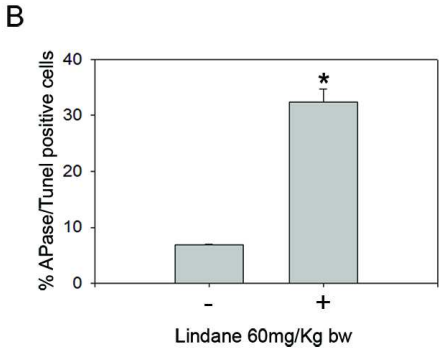

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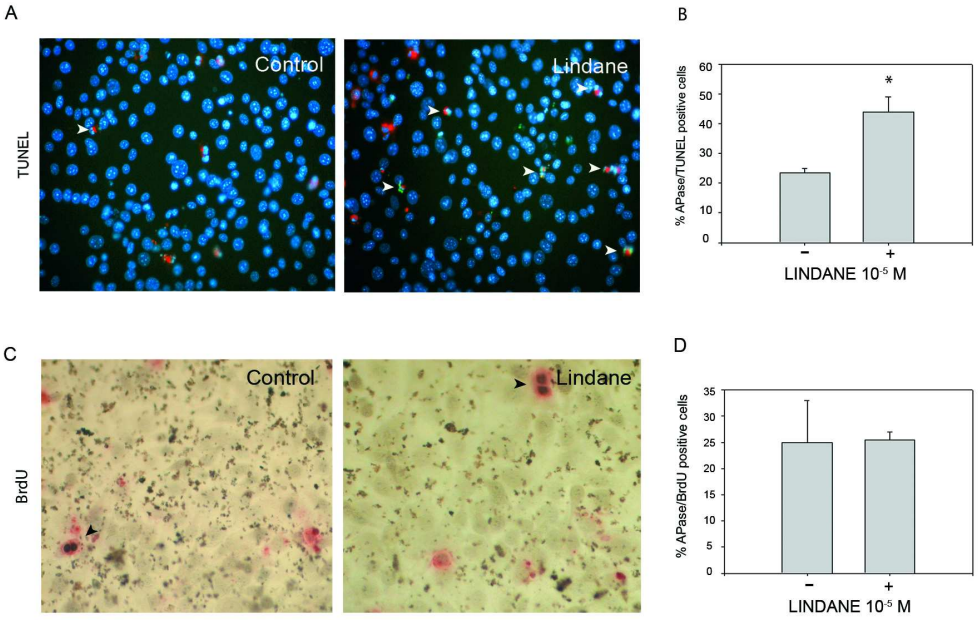

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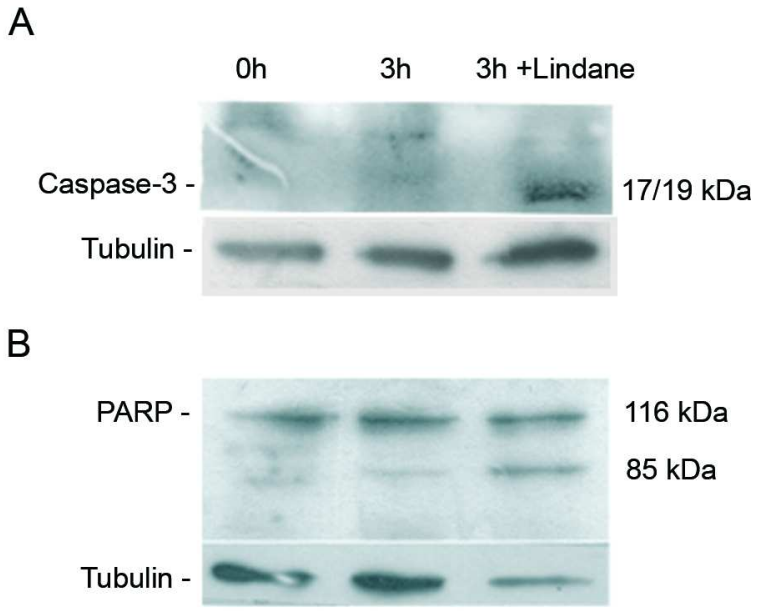

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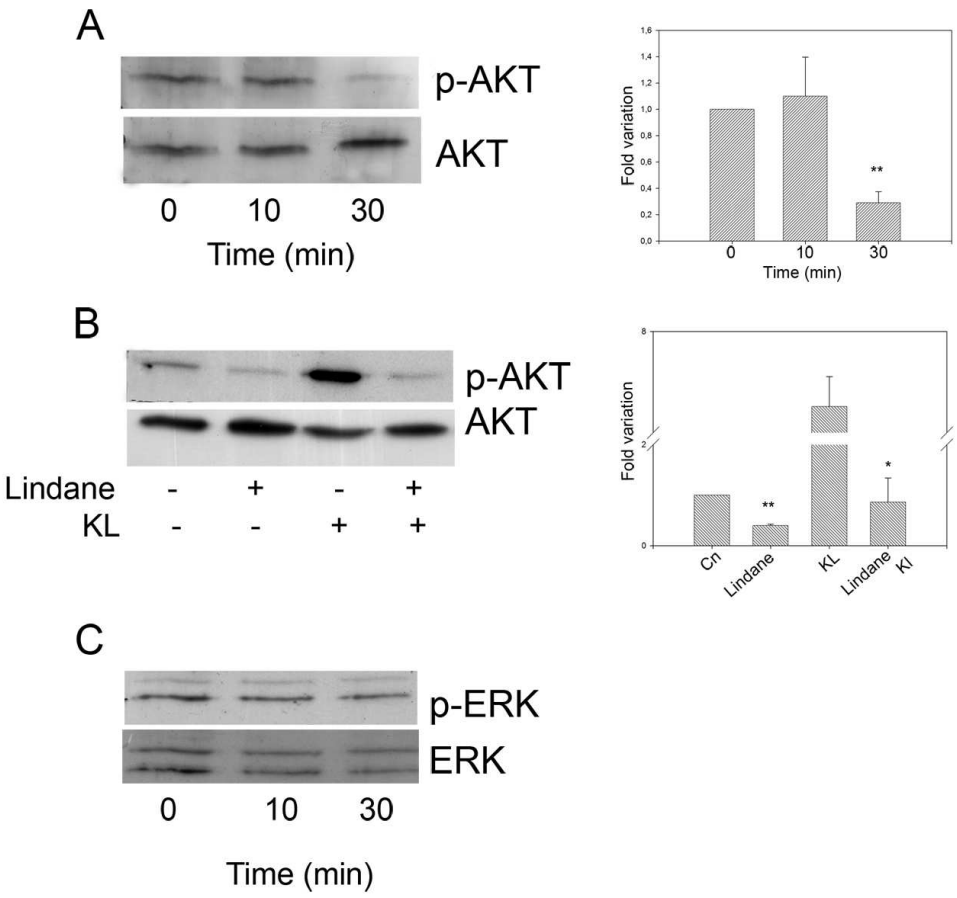

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SUPPLEMENTARY MATERIAL SUMMARY

TABLE 1: Number of PGCs in the testes of 12.5 dpc embryos exposed to lindane from 8.5 to 11.5 dpc

TABLE 2: Number of PGCs in the ovaries of 12.5 dpc embryos exposed to lindane from 8.5 to 11.5 dpc

FIG.1 Western blot analyses of the effect of lindane on AKT and ERK1/2 phosphorylation on somatic cells. Somatic cells were incubated in presence of 10^{-5}M lindane for the indicated time. The experiments were performed at least three times and typical blots are shown.

TABLE 1

Number of PGCs in the testes of 12.5 dpc embryos exposed to lindane from 8.5 to 11.5 dpc

Treatment	PGCs number/Embryo (mean±SEM)	N. Embryos	N. Litters
Olive oil	9207 ± 702	38	9
15 mg/Kg bw/day	7082 ± 784	17	4
30 mg/Kg bw/day	7181 ± 1058	19	4

TABLE 2

Number of PGCs in the ovaries of 12.5 dpc embryos exposed to lindane from 8.5 to 11.5 dpc

Treatment	PGCs number/Embryo (mean±SEM)	N. Embryos	N. Litters
Olive oil	10231 ± 598	37	9
15 mg/Kg bw/day	6564 ± 713	20	4
30 mg/Kg bw/day	6091 ± 739	25	4

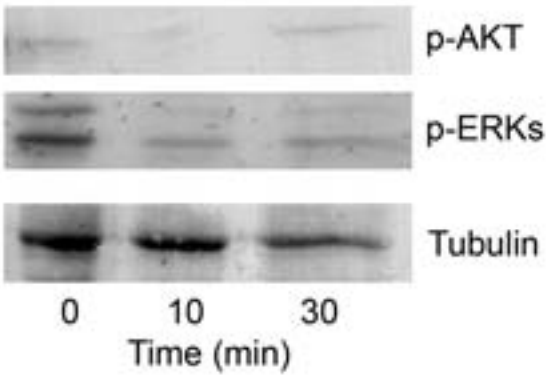

Figure 1

Estrogenic assay on putative Leydig cells from mouse embryonic testes

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ABSTRACT

We and others have reported that mouse embryonic testes from 12.5 dpc onward contain a subpopulation of somatic cells expressing estrogen receptor α (ERα). This marks testes as a possible target for estrogens and estrogenic compounds from early stages of embryo development. In the present study, in order to provide direct evidence of the possibility of estrogen effect on such cell population, we devised a protocol for their in vitro culture and transfection with a plasmid that contains the classical estrogen responsive element (ERE) or the alternative estrogen AP-1 responsive element upstream of the luciferase reporter gene (ERE-Luc and AP-1-Luc). StAR immunopositivity of the most part of the $ERα^+$ cells grown in culture and subjected to the estrogenic assay, allowed their identification as putative Leydig cells. Using the luciferase assay, we evaluated the estrogen activity of 17-ß estradiol (E2), the natural ligand of ERα, on such cells. For comparison, the same assay was carried out on a MCF-7 human breast cancer cell line expressing ERα. The results showed that 24 hr incubation in the presence of E2 resulted in a dose-dependent increase of ERE-Luc activity. Maximum induction of at concentration ERE-Luc activity from 1.7 to 3-fold in the putative Leydig cells was achieved with 10^{-8} M E2 and 2.3 to 5.7-fold in MCF-7 cells relative to vehicle-treated control. These effects were abolished when the antiestrogen 10^{-5} M ICI 182.80, was present in the assay. AP-1-Luc activity was less sensitive to E2 stimulation in both cell types (10^{-8} M E2: putative Leydig cells= 1.2 to 2.7-fold increase, MCF-7= 3-fold) and the effect of E2 was not abolished by ICI. Eventually to validate the assay with a xenoestrogen compound, we stimulated the transfected putative Leydig cells and MCF-7 cells with lindane (γ-HCH). Taken together these reported results represent the first evidence of a functional ERα pathway in putative Leydig cells from early stage of testis development and describe an in vitro assay that can be used as a tool to evaluate the activity of putative estrogenic compounds on mammalian embryonic testis.

INTRODUCTION

Estrogens are key regulators of growth, differentiation and function in a broad range of target tissues, including the male and female reproductive tracts, mammary gland, bone, brain and the cardiovascular system. The biological effects of estrogens are mediated through estrogen receptor α (ERα) and estrogen receptor β (ERβ), which belong to a large superfamily of nuclear receptors that act as ligand-activated transcription factors (for a review Mangelsdorf et al., 1995). The classical mechanism of activation of ERs depends on ligand binding to the receptors, after which the receptors dimerize and bind to estrogen response elements (EREs) located in the promoters of estrogen-responsive genes (McKenna et al., 1999; O'Malley et al., 2007). ERs may also regulate gene expression in the absence of DNA-binding by modulating the activities of other transcription factors via protein-protein interactions on DNA. This mechanism is referred to as cross-talk and is common for several nuclear receptors. For example, ligand-bound ERs upregulate and downregulate transcription from genes that contain AP-1 sites, binding sites for the Jun/Fos complex, in a manner that depends on the type of cells and the subtype of ERs.

In 1993, Sharpe and Skakkebaek formulated the hypothesis that the increased concentration in environment and in food of human made compounds that mimic the action of estrogens termed xenoestrogens, was responsible for increased disorder of the male reproductive function termed Testicular Dysgenesis Syndrome (TDS), including decrease of sperm count, increase in the incidence of testicular cancer and hypospadias and cryptorchidism (Sharpe and Skakkebaek, 1993). They then argued that the exposure to xenoestrogens during fetal and neonatal period cause this syndrome (Skakkebaek et al. 2001). A prerequisite of such hypothesis is that

reproductive tissues and gonads express functional ERs from early stages of development. Recent findings showed actually that ERs are expressed in reproductive tissues and gonads from early stages of embryo development in mammals, including humans (Saunders et al. 1998, Jefferson et al. 2000). In particular, we and others have reported that mouse embryonic testes from 12.5 dpc onward contain a subpopulation of somatic cells, identifiable mostly as Leydig cells, expressing ERα (Greco et al., 1992; Nielsen et al., 2000; Moe-Beherens et al., 2003). This marks testes as a possible target for estrogens and estrogenic compounds from early stages of development. Evidence of functional ERα in embryonic testicular cells, however, is lacking and no simple assay exists to evidence estrogenic activity of compounds on such cells. Aim of this work was to verify the presence of functional ERα in embryonic testicular cells using an ERE-and AP-1-Luc assay that could be used to quantify the activity of estrogenic compounds.

MATERIAL AND METHODS

Isolation and culture of somatic cells from 12.5 dpc mouse testes and of MCF-7 cell culture

All experiments were carried out in compliance with the ethical provisions enforced by the European Union and authorized by the National Committee of the Italian Ministry of Health.

CD1 female mice (Charles River, Italy) were mated with CD1 male mice and the detection of a vaginal plug the morning following mating was designated 0.5 day post coitum (dpc). Somatic cells (SCs) were obtained from the 12.5 dpc testes of CD-1 mice embryos following the method described in Pesce et al., 1995. About 5 x 10^4 testis SCs in 500µl of culture medium were seeded in each well of a 24-well Falcon dish. The culture medium consisted of phenol red-free DMEM with high glucose (GIBCO), containing non essential amino acids, 0.1 mM 2-mercaptoethanol, 2 mM glutamine, 0.25 mM pyruvate, 75 mg/L penicillin-G, 50 mg/L streptomycin, 0.5 mg/ml N-acetyl-L-cysteine (NAC) and 5% Horse Serum (HS) and 2.5% Fetal Calf Serum (FCS) (GIBCO). Culture was carried out in a humidified incubator at 37°C in 5% $C0_2$ air.

MCF-7 human breast cancer cell line was purchased from ATCC and cultured in DMEM w/o phenol red, 5 %fetal bovine serum (FBS), L-glutammine and antibiotics purchased by GibcoBRL/Invitrogen (Carlsbad, CA, USA).

Immunofluorescence

At the indicated time, cells were fixed in 4% paraformaldehyde, extensively washed and then permeabilized with Triton 2% for 5 min before incubation with the primary antibodies. Immunopositivity for StAR, MIS and Desmin was used as Leydig, Sertoli and myoid cell markers, respectively. Cells were incubated o/n at 4 °C with anti-StAR (1:250) (Santa Cruz cat. No. sc-25806), anti-MIS (1:50) (Santa Cruz cat. no. sc-6886), and with anti-Desmin (1:20) (Amersham). All antibodies were diluted in PBS with 1%BSA. Cell were washed and transferred in secondary antibodies, anti-rabbit (StAR), anti-goat (MIS) and anti-mouse (Desmin) were the added (1:500) for 1 hour. Negative controls, omitting the primary antibodies, were included. The IF for ERα detection was performed using anti-ERα antibody H222 (a kind gift by Prof. Green) following the protocol for permeabilized cells described in Norfleet et al. (1999). For double immunolocalization of $ER\alpha^+$ and $StAR^+$, after fixation and permeabilisation SCs were incubated with anti-StAR o/n at 4°C and incubated with secondary anti-rabbit made in donkey. The SCs were the incubated with the anti-ERα for 2h at RT and then incubated with anti-rat made in rabbit.

RT-PCR for ERα and ERβ

Total RNA was extracted from cultured testis SCs and from MCF-7 with RNeasy minikit (Qiagen) in accordance to the manufacturer's instructions. First-strand cDNA synthesis was performed as follows: 200 ng total RNA was reverse transcribed by 50 U of Superscript™II (Invitrogen) using 50 ng random hexamers, in the presence of 0.5 μM deoxynucleotide triphosphates in a final volume of 20 ul. DNA contamination or PCR carry over controls were performed omitting reverse transcriptase during

reverse transcription. The reaction mixture was incubated for 1 h at 42°C, then heat denatured for 15 min at 75 °C 3 ul of the obtained cDNA was used to amplify ERα, ERβ.

Primer sequences were:

mERα: 5'-ACCATTGACAAGAACCGGAG-3'

5'-ATAGATCATGGGCGGTTCAG-3'.

mERβ: 5'-TCTGCATAGAGAAGCGATGA-3'

5'-GGCATTCTACAGTCCTGCTG-3'

hERα 5'-AGACATGAGAGCTGCCAACC-3'

5'-GCCAGGCACATTCTAGAAGG-3'

hER-β 5'-TCACATCTGTATGCGGAACC-3'

5'-CGTACCACTTCCGAAGTCGG-3'

Reactions were performed using the following reagents: 0.5uM of primers, Taq polymerase (2U/tube) in a finale volume of 20ul. The amplification consisted of: 35 cycles at 95°C for 1min, 58°C for 1min, 72°C for 1min for mERα ; 35 cycles 95°C for 1min, 62°C for 1min, 72°C for 2 min for mERβ; 35 cycles 95°C for 1min, 58°C for 30 sec, 72°C for 30 s for hERα/ hERβ. Adult uterus and ovary served as positive controls for mERα and mERβ respectively, while LNCaP as a positive control of hERβ

ERE-and AP-1-Luc assay

After 24 h of culture, testis SCs were transfected with 1 µg of an ERE- or AP-1 dependent luciferase reporter gene (3XERE-TATA-Luc, provided by Prof. McDonnell; AP-1-Luc, Stratagen, cat. n. 219074-51) and Renilla luciferase reporter

gene (10 ng) as an internal transfection control using the TransFastTM Transfection Reagent (Promega Italia SRL, Milan, Italy) according to the manufacturer's instructions. In preliminary experiments, using pEGFP-C1 construct (Clonetech), we estimated that under the conditions used by us the efficiency of transfection of the putative Leydig $StAR^+$ $ER\alpha^+$ cells (see, Results) was around 30%.

After 24h of culture, transfected cells were extensively washed and starved for 24h in the same culture medium as above but without serum. Eventually, cells were incubated in the presence of 17-β-estradiol (E2) or lindane (γ-HCH) both purchased from Sigma with or without the antiestrogen ICI 182780 (ICI) (Tocris) at the indicated concentrations for additional 24h. Control cultures were treated with vehicle alone (1: 1000 v/v ethanol). At the end of the incubation, cells in each well were harvested and lysed in 250 µl passive lysis buffer (PLB) (Dual-Luciferase Reporter Assay System, Promega Italia SRL, Milan, Italy) for 15 min at room temperature. Cell lysates were cleared for 30 sec by centrifugation at top speed in a refrigerated microcentrifuge and transferred to a fresh tube prior to reporter enzyme analysis. Ten microliter of cell lysates from each well were mixed with 100 µl of Luciferase Assay Reagent II (LAR II, Promega), and the ERE-firefly luciferase activity was determined using a biocounter luminometer. For the assessment of the Renilla luciferase activity, 100 µl of Stop & Glo^R Reagent were added to the same sample.

Statistics

All experiments were replicated at least three times. The means were tested for homogeneity of variance and analyzed by ANOVA. The level of significance was set at $P \leq 0.05\%$ and $P \leq 0.01\%$.

RESULTS AND DISCUSSION

RT-PCR analyses carried out to verify the expression of ER transcripts on testis SCs after 3 day of culture and MCF-7 cells used as control, confirmed the expression of ERα and not of ER β in either cell types (Fig.1).

Using immunofluorescence with antibodies specific for the three main somatic cell types of the testis, namely anti-MIS for Sertoli cells, anti-Desmin for myoid cells and anti-StAR for Leydig cells, we next estimated that under our culture conditions the percentages of these cell types after three days of culture were 12±2.7% (mean of three experiments ± standard error) for Sertoli cells, 20 ± 1.8% of myoid cells and 50 ± 6.5 % of Leydig cells (Fig. 2). Moreover, while virtually all $StAR^+$ cells were $ER\alpha^+$ (Fig. 2D), about 10% of the $ER\alpha^+$ cells were $StAR^-$. Overall these observations indicated that around 60% of the cells subjected to the ERE- and AP-1-Luc assay expressed ERα and that most of these latter were putative Leydig cells.

In order to verify the functionality of ERα in the cultured testis SCs, we first transfected with ERE- and AP1-Luc plasmids and then after 24 hr of starvation in serum-free culture medium, performed cell stimulation with 1-100 nM E2 for further 24 h. The results of the Renilla Luciferase assay for ERE-Luc show a dose-response effect of E2 and a maximal response of 2.8 ± 0.4% -fold stimulation at 10nM E2 relative to vehicle-treated controls (Fig. 3A). Similar results were obtained when Renilla Luciferase assay were carried out on MCF-7 cell line (Fig. 3B). In accord to previous results (Balaguer et al., 1995) maximal stimulation by 10nM E2 reached an higher value of 5.7 ± 0,7%-fold. In both cell type stimulation by 10nM E2 was abolished by the presence of 10 µM ICI.

The Renilla Luciferase assay for AP-1-Luc carried out in SCs and MCF-7 cells showed that in both cell types AP-1 site was less responsive to the hormone. In fact, we observed only 1.76 ± 0.23%-fold and 3 ± 0.15 -fold stimulation by 10nM E2 in SCs and MCF-7 cells, respectively. Interestingly, ICI failed to inhibit this effect (Fig. 4).

While the low activity of the AP-1-Luc reporter is in line with the notion that ERα is a weaker activator of AP-1 in comparison to ERβ, the lack of the ICI inhibitory effect can be explained by the fact that ERs can enhance AP-1 activity through two different mechanisms (for a review, see Kushner et al., 2000). It is well established that ER action at classical EREs is mediated by transactivation functions.

ERα contains two separate transactivation functions, AF-1 and AF-2, which synergize strongly to give the overall level of estrogen response. AF-2 of both receptors consists of a small hydrophobic patch on the surface of the estrogen-liganded LBD. ERs can enhance AP-1 activity in a manner that requires ER transactivation functions, but not the ER-DBD or with a mechanism that is independent of ER activation functions, but does require the ER-DBD. This AF-1 independent pathway is activated by antiestrogens, especially those with high antiestrogenic potential like ICI (for a review, see Kushner et al., 2000).

Finally, to further validate our assay, we stimulated putative Leydig cells and MCF-7 cell with a typical xenoestrogen compound lindane at 10 µM, a concentration able to elicit estrogen-dependent effect in MCF-7 cells (Steinmetz et al., 1996). The results reported in Fig. 5, show that in testis SCs lindane stimulated in a ICI-dependent manner ERE-Luc at similar level than E2 (2.8 ± 0.6%-fold induction) while it failed to stimulate AP1-Luc expression. In MCF-7 cells, in line with previous results (Balaguer et al., 2001), this compound exerted a weak action on ERE-Luc (1.9 ± 0.36%) about 2-fold lower than E2. Moreover, in such cells, lindane caused AP-1-Luc stimulation similar to that of E2 (about 3 times).

On the whole the comparison between putative embryonic Leydig cells and MCF-7 allows to evidence similar ERE and AP-1 promoter element responsiveness to E2. Relatively to ERE-luc, however, Leydig cells appeared, however, more sensitive to lindane. In this regard it is to be noted that while the estrogen responsiveness of MCF-7 cell in term of proliferation and apoptosis or gene expression is well documented (Detze et al., 1999; Jouer et al., 2000; Yoshida et al., 2002), little is know about the effects of estrogens and xenoestrogens on embryonal Leydig cells. Several studies have reported various effects of the prenatal exposure to estrogens and estrogenic compounds including environmental xenoestrogens on rodent testis development (for a review, see Delbès et al., 2006; 2007). For example in the rat in utero exposure from 11.5 to 15.5 dpc to E2 and various xenoestrogens alter expression of steroidogenic factor-1 (SF-1) in the foetal testis (Majdic et al., 1997) while bisphenol A (BPA) exposure on day 6-21 of gestation leads to stimulatory effects on Sertoli cell number. In culture E2 and diethylstilbestrol (DES) caused various alteration in Leydig cells, Sertoli cells and gonocytes (Lassurguère et al., 2003). In the mouse, DES inhibits the expression of StAR protein in foetal testis mouse fetal testis (Guyot et al., 2004). Moreover using ERα knockout mice, it has

been recent reported that endogenous estrogens inhibit fetal Leydig cell development (Delbes et al., 2004) and that ERα is a major contributor to estrogen-mediated testis dysgenesis and cryptorchidism (Cederroth et al., 2008).

In this regard the results presented in the present paper are particular relevant since they represent the first direct evidence of the existence of functional ERα-mediated pathway in putative Leydig cells leading to activation of ERE and at lower extend of AP-1 promoter element from early stage of testis development. Moreover, the method described here represent a simple in vitro assay that can be used to evaluate estrogenic activity of compounds on mammalian embryonic testis. Further studies are underway to investigate the effect of estrogens and xenoestrogens on biological functions and gene expression in the putative Leydig cells in culture.

Acknowledgements

The present study was supported by European Community project "GENDISRUPT" (QLK4-CT-2002-02403) and Ministero del Lavoro e della Previdenza Sociale grant n. 1650.

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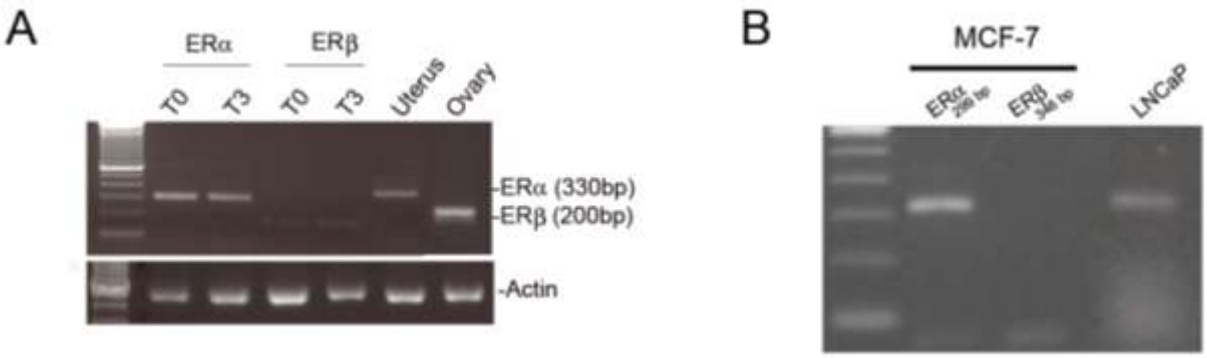

Figure 1. Expression of ERα and ERβ transcripts in cultured testis SCs and MCF-7. A. RT-PCR analyses on testis SCs cultured for 0 and 3 days from 12.5 dpc testes and on **B**. MCF-7 cells showed the presence in both cell types of ERα and not ERβ. Uterus was used ad positive control for mERα and ovary as control for mERβ. LNCaP was used as control for hERβ.

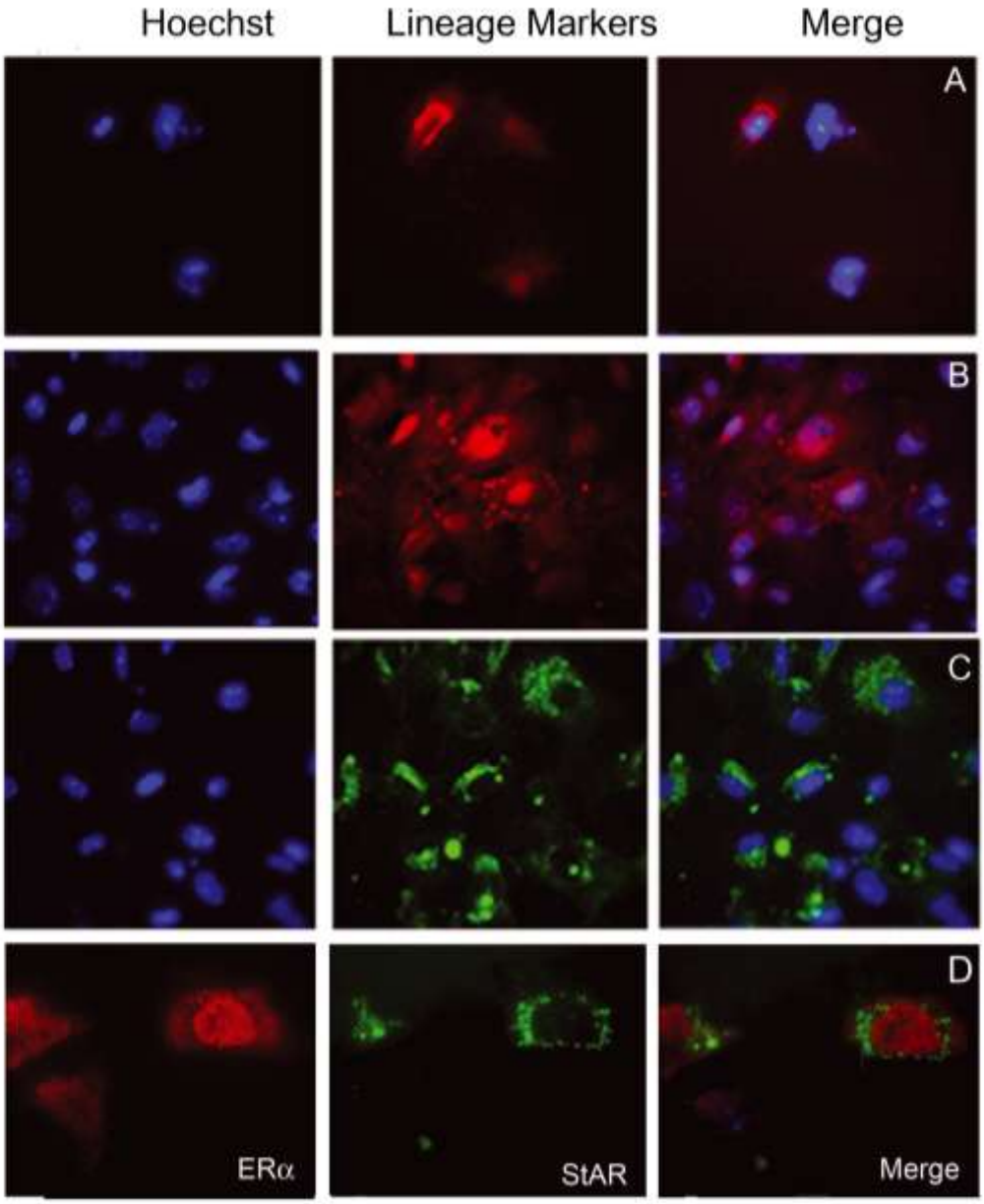

Figure 2. Immunofluorescence on testis SCs after 3 days. Representative fields of SCs stained as follows: Desmin (A) for myoid cells, StAR (B) for Leydig cells, MIS (C) for Sertoli cells and (D) double immunolocalization of $ER\alpha^{+}$ and $StAR^{+}$.

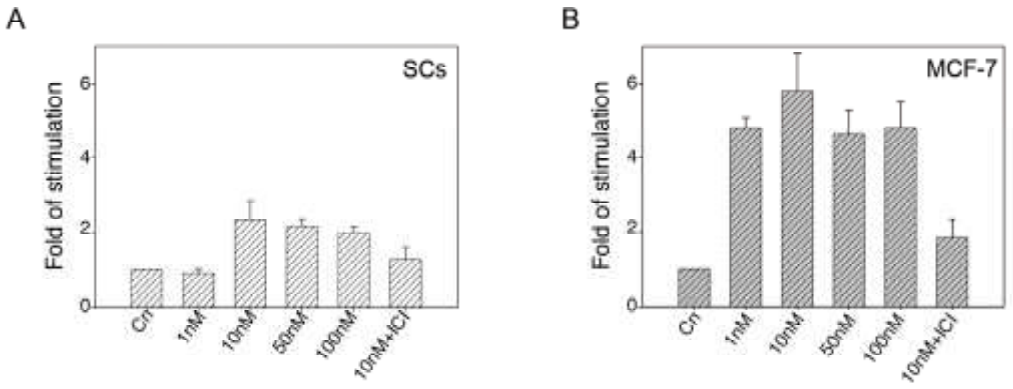

Figure 3. ERE-luc activity in testis SCs and MCF-7 stimulated by 17-β-estradiol. **A**. Testis SCs and **B**. MCF-7 cells. Data represent the mean±SE of at least three separate experiments. Fold increase for 10, 50 and 100nM was statistically significant, $p < 0.05$.

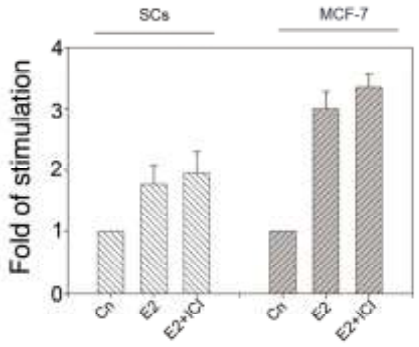

Figure 4. AP1-luc activity in testis SCs and MCF-7 cells stimulated by 17-β-estradiol. Data represent the mean ± SE of at least three separate experiments. Fold increase for E2 and E2+ICI was statistically significant, $p < 0.05$.

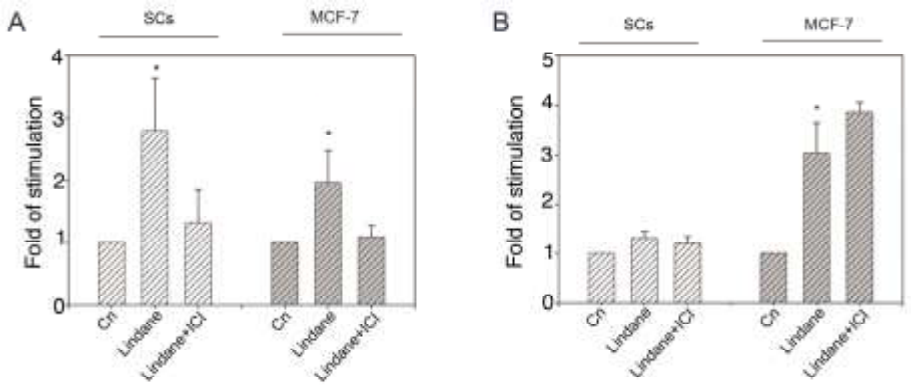

Figure 5. ERE-luc and AP1-luc activity in testis SCs and MCF-7 cells of lindane. **A**. ERE-luc activity in SCs and MCF-7 cells. **B**. AP-1-luc activity in SCs and MCF-7 cells. * = $p < 0.05$

Effect of 17-ß-estradiol and lindane on the expression of cell cycle genes of somatic cells of foetal testis

INTRODUCTION

The primary mechanism of action of estrogens is mediated by estrogen receptors α and β (ERα, ERβ). In response to estrogen, both receptors form homo or heterodimers and interact with estrogen responsive elements ERE within promoters region of estrogen-responsive genes to stimulate gene transcription (Klinge et al., 2001). Other action of estradiol involve other transcription factor complexes like Fos/Jun (AP-1 response elements) or SP-1 (GC-rich motifs). These events are generally termed "estrogen genomic effect" and are involved only to the involvement of gene transcription and translation (Webb et al., 1999; Safe et al., 2001).

ERα is expressed very early during testis development (Greco et al., 1992) and it is expressed at fetal stage by Leydig and myoid cells and expressed until the birth in these cells. ERβ protein is present at 16 dpc in three main types (Leydig, Sertoli, gonocytes) of testicular cells in the rat but is found exclusively in the gonocytes in the mouse (Saunders et al. 1998, Jefferson et al. 2000).

In several species including mammals, the expression of ERs from early stages of testis development (Greco et al., 1992; Saunders et al. 1998, Jefferson et al. 2000) makes embryonic and fetal testes possible target for estrogens and estrogenic compound. In the mouse, testes and adrenals become able to produce estrogens only after 13.5 dpc, so that before this time testes receive estrogens only from the mother. Actually, in mammals there

is not evidence that the normal level of maternal estrogens play a role during early testis development and it is not known if exposure of mother to estrogens and estrogenic compound may alter testis development in the embryo.

In previous investigations reported in other sections of the present thesis, we found that a subpopulation of the somatic cells of the mouse embryonic testes, mostly identifiable in Leydig cells, express ERα and is responsive to E2 in a ERE-luc, Ap1-luc assay. Since cell proliferation is one of the most common estrogen target process the aim of this part of work was to evidence the effect of E2 and lindane, a prototype of ED on the levels of mRNA transcripts of cell-related genes in somatic cells of the embryonic testes. To this aim, we used a focused cDNA array containing 112 cell cycle-related genes (superarray) on cultured somatic cells of mouse fetal testis (12.5 dpc) expressing estrogen receptor α (ERα). In a parallel study, we used the same assay to evaluate estrogenic action on the same cells by lindane.

MATERIAL AND METHODS

Isolation and culture of testis somatic cells

CD1 female mice (Charles River, Italy) were mated with CD1 male mice and the detection of a vaginal plug the morning following mating was designated 0.5 day post coitum (dpc). Somatic cells (SCs) were obtained from the testes of 12.5 dpc CD-1 mice embryos following the methods of immunomagnetic separation described in Pesce et al.,1995.

SCs from 10 testes were seeded in each well of a 24-well Falcon dish in 500µl of culture medium. The culture medium consisted of phenol red-free DMEM with high glucose (Gibco), containing non essential amino acids, 0.1 mM 2-mercaptoethanol, 2 mM glutamine, 0.25 mM pyruvate, 75 mg/L penicillin-G, 50 mg/L streptomycin, 0.5 mg/ml N-acetyl-L-cysteine (NAC) and supplemented with 0,1% BSA (Sigma).

The cells were grown for 24 h under control condition (1:1000 EtOH) or in continuous presence of 10^{-8} M estradiol or 10^{-5}M lindane.

Immunoblotting for ERα

For Western blotting analysis, cells were lysed in a solution containing 50 mM Tris, 150 mM NaCl, 20 mM EDTA, 1% deoxicholate, 0.1% SDS, 1 mM PMSF, 20 µ/ml leupeptin and 1 mM Na vanadate. Proteins (about 30 µg)) were subjected to 10% SDS/PAGE electrophoresis. After transfer to Hybond C nitrocellulose membranes, blots were incubated o/n at 4°C with rat α-ERα (1:500, gently gift by Prof. Green). Secondary anti-rat peroxidase-conjugated antibody (Amersham) was incubated with the membranes for 1 h at room temperature, at 1:10000 dilution in TBS/Tween 20. Immunostained bands were detected by chemiluminescent method (Amersham).

RNA extraction and amplification

Total RNA from SCs cultured for 24 h following the culture condition described above were extracted using the Rneasy micro kit QIAGEN accordin to the manufacturer's protocol. Approximately 100ng total RNA were converted into double-stranded cDNA using a cDNA synthesis kit (TargetAmp^{TM} 1-Round Aminoallyl-aRNA Amplification Kit 101, EPICENTRE) with a special oligo(dT)_24 primer containing a T7 RNA promoter site added 5' to the poly(T) tract. After the cDNA synthesis high yields of Aminoallyl-aRNA (AA-aRNA) are produced in a rapid in vitro transcription reaction (TargetAmpTM T7 kit).

cDNA Array analysis

After purification, AA-aRNA was biotinylated using the Biotin-X-X-NHS (according to the manufacturer's instruction (EPICENTRE) for preparing labeled target for hybridization to microarray. 10 μg of the biotin-labeled cRNAs probes were hybridized to the Mouse Oligo Cell Cycle Microarray (OMM-020 Mouse Cell Cycle oligo GEArrays"; SuperArray) at 60°C overnight according to the manufacturer's protocol. After washing and blocking array membranes, alkaline phosphatase-conjugated streptavidin was allowed to bind and the CDP-Star substrate (SuperArray, Inc., Frederick, MD, USA) chemiluminescence detected by exposure to x-ray film. The resulting film was scanned and quantified using a GEArray Expression Analysis Suite (GEASuite) software (SuperArray). All signal intensities of genes were first corrected subtracting the background (PUC18 plasmid) and normalized to the housekeeping genes. The normalized mean expression of each spotted gene was expressed as the ratio 17-β-Estradiol/EtOH or Lindane/EtOH in provided scatter plot represented in Fig. 2 and 3, respectively. Changes in gene expression were illustrated as a fold increase/decrease. The cut-off induction determining expression was ≥2.0

or ≤−2.0 fold changes. Genes which suited both above criteria were considered to be upregulated or downregulated. The experiments were repeated tree times.

Analysis of the SCs proliferation in culture

Cultured SCs were incubated in the presence of BrdU for the last 3h of culture (labeling reagent diluted 1:100, according to the instructions for the cell proliferation kit; Amersham, Buckinghamshire, England). At the end of 24h of culture SCs incubation SCs were then fixed with parafolmaledeyde 4% for 15 min at RT washed and incubated for 1 h with mouse anti-BrdU monoclonal antibody peroxidase-conjugated secondary (cell proliferation kit, Amersham) at RT. Finally, cells were stained with DAB (cell proliferation kit, Amersham). The number of BrdU-labeled cells relative to the total number of cells per field of vision was determined. A minimum of 5 fields each containing at least 50 cells were counted.

RESULTS

Presence of ERα-expressing cells in cultured SCs

In order to verify the presence of ERα expressing cells in testes SCs cultured for 24h, we used immunofluorescence and western blot. Both methods confirmed the presence of ERα expressing cells in all culture conditions without significant difference (Fig.1).

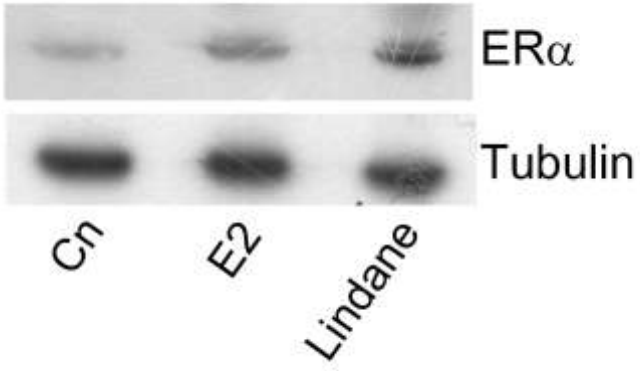

Figure 1. W. Blot of ERα expression in testes SCs. SCs cultured for 24h in continuous presence of vehicle (1:1000 EtOH) as a control or 10^{-8}M 17–β-estradiol (E2) or 10^{-5}M lindane**.** 30µg of cell lysates were analysed using the antibody H222 (anti ERα antibody). The same membrane was reprobed with mouse anti-tubulin. Experiments were performed 2 times with similar results; a typical blot is shown.

Effect of E2 and lindane on the transcription level of cell cycle genes testes SCs

To identify genes of cell cycle affected by exposure to E2 and lindane in testes SCs, we compared the level of mRNA transcripts in such cells cultured for 24h in the presence of either vehicle (EtOH) or 10^{-8}M E2 and 10^{-5}M lindane, two concentrations found in previous ERE-luc and AP1-luc assay effective in stimulating the expression of these probes.

In summary, the c-DNA-array analyses showed that, among the 112 genes (Table 3), 74 did not have significant level of transcripts in the cell population examined (AP threshold fixed to 1,2), of the remaing 38 genes, 20 genes were down-regulated after E2

treatment (Table 1) (Fig. 2) However only the expression level of 10 genes, namely *Cdkn1a* (*p21*), *Ddit3*, *Cdkn2a* (*p16)*, *CycB2*, *Cks1b*, *E2f5*, *Ccnc*, *Ccne1*, *Abl1*, *Cdc25a*, resulted downregulated in a statistically manner. It is noted that among these genes, 3 (*Cdkn1a, Cdkn2a, Ddit3)* are cell-cycle inhibitors while the other 7 (*CycB2*, *Cks1b*, *E2f5*, *Ccnc*, *Ccne1*, *Abl1*, *Cdc25a)* are generally expressed at high level in proliferation cells. Thus supporting a prevalent anti-proliferative effect of E2 on testis SCs.

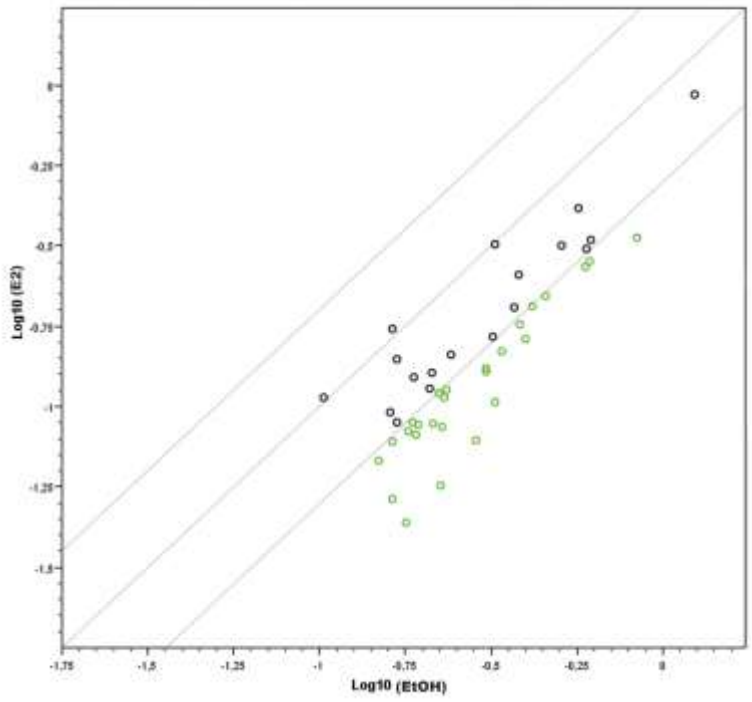

Figure 2. Scatter plot analysis representing the comparison of the log10-scaled expression of testis SCs exposed to E2 or EtOH. Black circles represent genes whose expression profiles were found to be not affected by E2 exposure while green circles represent genes whose expression was downregulated by E2 exposure (≥2-fold change).

Table 1. Top 20 genes specifically downregulated in 12.5 dpc male somatic cells by E2 exposure (>2-fold)

ID	*Gene symbol*	*Gene name/description*	*Fold Change*	*P-value*
NM_009594	**Abl1**	**V-abl Abelson murine leukemia oncogene 1**	**0,47**	**0.046**
NM_009685	Apbb1	Amyloid beta (A4) precursor protein-binding, family B, member 1	0,30	
NM_009686	Apbb2	Amyloid beta (A4) precursor protein-binding, family B, member 2	0,11	
NM_009765	Brca2	Breast cancer 2	0,28	
NM_023813	Camk2d	Calcium/calmodulin-dependent protein kinase II, delta	0,13	
NM_007630	**Ccnb2**	**Cyclin B2**	**0,18**	**0.04**
NM_016746	**Ccnc**	**Cyclin C**	**0,19**	**0.02**
NM_007632	Ccnd3	Cyclin D3	0,36	
NM_007633	**Ccne1**	**Cyclin E1**	**0,26**	**0.036**
NM_007658	**Cdc25a**	**Cell division cycle 25 homolog A (S. cerevisiae)**	**0,09**	**0.026**
NM_016742	Cdc37	Cell division cycle 37 homolog (S. cerevisiae)	0,24	
NM_009862	Cdc45l	Cell division cycle 45 homolog (S. cerevisiae)-like	0,22	
NM_007669	**Cdkn1a**	**Cyclin-dependent kinase inhibitor 1A (P21)**	**0,32**	**0.034**
NM_009876	Cdkn1c	Cyclin-dependent kinase inhibitor 1C (P57)	0,42	
NM_009877	**Cdkn2a**	**Cyclin-dependent kinase inhibitor 2A**	**0,30**	**0.049**
NM_016904	**Cks1b**	**CDC28 protein kinase 1b**	**0,42**	**0.034**
NM_007837	**Ddit3**	**DNA-damage inducible transcript 3**	**0,34**	**0.005**
NM_007892	**E2f5**	**E2F transcription factor 5**	**0,35**	**0.016**
NM_010564	Inha	Inhibin alpha	0,40	
NM_008563	Mcm3	Minichromosome maintenance deficient 3 (S. cerevisiae)	0,39	
NM_008628	Msh2	MutS homolog 2 (E. coli)	0,32	
NM_009009	Rad21	RAD21 homolog (S. pombe)	0,21	

Genes shown in bold are genes whose change resulted statistically significant.

In testis SCs exposed to lindane the level of transcripts of 72 genes were not significative while of the remaing 40, the transcripts of 5 genes resulted down-regulated and 4 up-regulated (Table 2) (Fig.3). Statistical analysis found, however, that only *Mcm7* and *Rad9* two genes, generally active in the S phase, were down and up regulated, respectively, in a statistically significant manner. On the whole these results indicate that lindane at last at this tested concentration exerts different and much less effective genomic effects on testis SCs than E2.

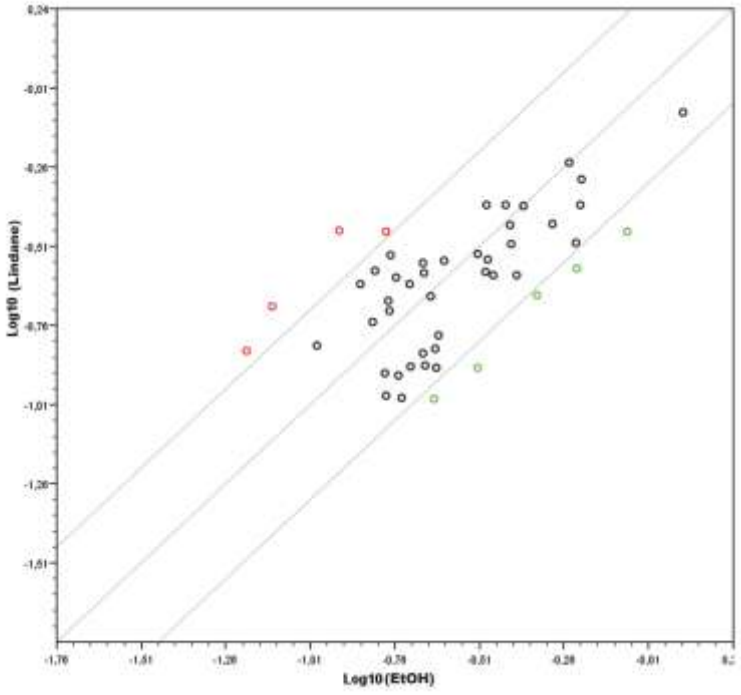

Figure 3. Scatter plot analysis representing the comparison of the log10-scaled expression signal of testis SCs exposed to lindane or EtOH. Black circles represent genes whose expression profiles were not affected by lindane exposure while green and red circles represent genes whose expression were downregulated and upregulated, respectively (≥2-fold change).

Table 2. Top 9 genes specifically deregulated in 12.5 dpc male somatic cells by lindane exposure (>2-fold)

ID	*Gene symbol*	*Gene name/description*	*Fold Change*	*P-value*
NM_009594	Abl1	V-abl Abelson murine leukemia oncogene 1	0,2	
NM_009685	Apbb1	Amyloid beta (A4) precursor protein-binding, family B, member 1	0,3	
NM_007632	Ccnd3	Cyclin D3	0,30	
NM_009876	Cdkn1c	Cyclin-dependent kinase inhibitor 1C (P57)	0,24	
NM_134448	Dst	Dystonin	0,37	
NM_008568	**Mcm7**	**Minichromosome maintenance deficient 7 (S. cerevisiae)**	**0,37**	**0.026**
NM_011237	**Rad9**	**RAD9 homolog (S. pombe)**	**4,59**	**0.014**
NM_009391	Ran	RAN, member RAS oncogene family	2,29	
NM_011249	Rbl1	Retinoblastoma-like 1 (p107)	3,33	

Genes shown in bold are genes found to be statistically significant

E2 and lindane do not affect testis SCs proliferation in culture

In order to verify possible E2 and lindane effect on testis SCs proliferation, we performed BrdU incorporation assay in SCs treated with E2 or Lindane for 24h in culture. This assay did not reveal any evident positive or negative effect of E2 and lindane on the whole SCs. These experiments should be repeated using double staining for ERα and BrdU to achieve more precise information. (Fig.4).

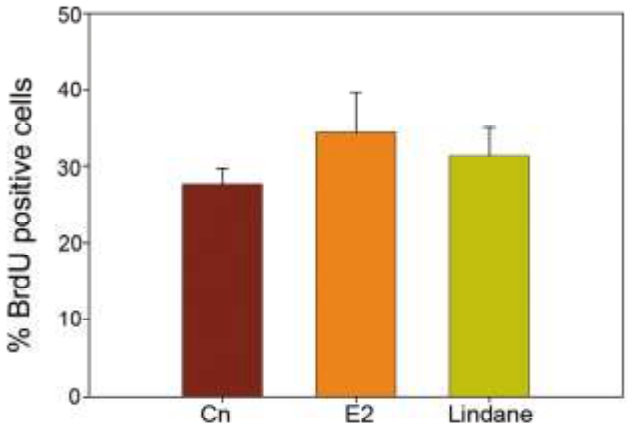

Figure 4. Analysis of BrdU incorporation in SCs obtained from .12.5 dpc testes and cultured for 24h in presence of 10^{-8}M 17-β-estradiol and 10^{-5}M lindane. BrdU positive cells are reported as means ± SD of three indipendent experiments.

Table 3. Complete list of cell-cycle genes analyzed

ID	*Symbol*	*Description*
NM_001001303	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
NM_009594	Abl1	V-abl Abelson murine leukemia oncogene 1
NM_021515	Ak1	Adenylate kinase 1
NM_009685	Apbb1	Amyloid beta (A4) precursor protein-binding, family B, member 1
NM_009686	Apbb2	Amyloid beta (A4) precursor protein-binding, family B, member 2
NM_007499	Atm	Ataxia telangiectasia mutated homolog (human)
NM_009764	Brca1	Breast cancer 1
NM_009765	Brca2	Breast cancer 2
NM_177407	Camk2a	Calcium/calmodulin-dependent protein kinase II alpha
NM_007595	Camk2b	Calcium/calmodulin-dependent protein kinase II, beta
NM_023813	Camk2d	Calcium/calmodulin-dependent protein kinase II, delta
NM_178597	Camk2g	Calcium/calmodulin-dependent protein kinase II gamma
NM_009810	Casp3	Caspase 3
NM_007628	Ccna1	Cyclin A1
NM_009828	Ccna2	Cyclin A2
NM_172301	Ccnb1	Cyclin B1
NM_007630	Ccnb2	Cyclin B2
NM_016746	Ccnc	Cyclin C
NM_007631	Ccnd1	Cyclin D1
NM_009829	Ccnd2	Cyclin D2
NM_007632	Ccnd3	Cyclin D3
NM_007633	Ccne1	Cyclin E1
NM_009830	Ccne2	Cyclin E2
NM_007634	Ccnf	Cyclin F
NM_007658	Cdc25a	Cell division cycle 25 homolog A (S. cerevisiae)
NM_023117	Cdc25b	Cell division cycle 25 homolog B (S. cerevisiae)
NM_016742	Cdc37	Cell division cycle 37 homolog (S. cerevisiae)
NM_009862	Cdc45l	Cell division cycle 45 homolog (S. cerevisiae)-like
NM_016756	Cdk2	Cyclin-dependent kinase 2
NM_009870	Cdk4	Cyclin-dependent kinase 4
NM_025876	Cdk5rap1	CDK5 regulatory subunit associated protein 1
NM_030248	Cdk5rap3	CDK5 regulatory subunit associated protein 3
NM_007669	Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21)
NM_009875	Cdkn1b	Cyclin-dependent kinase inhibitor 1B (P27)
NM_009876	Cdkn1c	Cyclin-dependent kinase inhibitor 1C (P57)
NM_009877	Cdkn2a	Cyclin-dependent kinase inhibitor 2A
NM_007670	Cdkn2b	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)
NM_009878	Cdkn2d	Cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)
NM_007691	Chek1	Checkpoint kinase 1 homolog (S. pombe)
NM_016904	Cks1b	CDC28 protein kinase 1b

NM_007837	Ddit3	DNA-damage inducible transcript 3
NM_134448	Dst	Dystonin
NM_007891	E2f1	E2F transcription factor 1
NM_177733	E2f2	E2F transcription factor 2
NM_010093	E2f3	E2F transcription factor 3
NM_148952	E2f4	E2F transcription factor 4
NM_007892	E2f5	E2F transcription factor 5
NM_033270	E2f6	E2F transcription factor 6
NM_007836	Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha
NM_008086	Gas1	Growth arrest specific 1
NM_008087	Gas2	Growth arrest specific 2
NM_019925	Gpr132	G protein-coupled receptor 132
NM_008316	Hus1	Hus1 homolog (S. pombe)
NM_010564	Inha	Inhibin alpha
NM_010578	Itgb1	Integrin beta 1 (fibronectin receptor beta)
XM_110503	Macf1	Microtubule-actin crosslinking factor 1
NM_019499	Mad2l1	MAD2 (mitotic arrest deficient, homolog)-like 1 (yeast)
NM_008564	Mcm2	Minichromosome maintenance deficient 2 mitotin (S. cerevisiae)
NM_008563	Mcm3	Minichromosome maintenance deficient 3 (S. cerevisiae)
NM_008565	Mcm4	Minichromosome maintenance deficient 4 homolog (S. cerevisiae)
NM_008566	Mcm5	Minichromosome maintenance deficient 5, cell division cycle 46 (S. cerevisiae)
NM_008567	Mcm6	Minichromosome maintenance deficient 6 (MIS5 homolog, S. pombe) (S. cerevisiae)
NM_008568	Mcm7	Minichromosome maintenance deficient 7 (S. cerevisiae)
NM_010786	Mdm2	Transformed mouse 3T3 cell double minute 2
XM_133912	Mki67	Antigen identified by monoclonal antibody Ki 67
NM_018736	Mre11a	Meiotic recombination 11 homolog A (S. cerevisiae)
NM_008628	Msh2	MutS homolog 2 (E. coli)
NM_134092	Mtbp	Mdm2, transformed 3T3 cell double minute p53 binding protein
NM_033597	Myb	Myeloblastosis oncogene
NM_010892	Nek2	NIMA (never in mitosis gene a)-related expressed kinase 2
NM_011848	Nek3	NIMA (never in mitosis gene a)-related expressed kinase 3
NM_016791	Nfatc1	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
NM_010928	Notch2	Notch gene homolog 2 (Drosophila)
NM_181345	Npm2	Nucleophosmin/nucleoplasmin 2
NM_011045	Pcna	Proliferating cell nuclear antigen
NM_022889	Pes1	Pescadillo homolog 1, containing BRCT domain (zebrafish)
NM_013630	Pkd1	Polycystic kidney disease 1 homolog
NM_008861	Pkd2	Polycystic kidney disease 2
NM_008885	Pmp22	Peripheral myelin protein
NM_016910	Ppm1d	Protein phosphatase 1D magnesium-dependent, delta isoform
XM_356255	Ppp2r3a	Protein phosphatase 2 (formerly 2A), regulatory subunit B, alpha
NM_008913	Ppp3ca	Protein phosphatase 3, catalytic subunit, alpha isoform

NM_013637	Prm1	Protamine 1
NM_008933	Prm2	Protamine 2
NM_011233	Rad17	RAD17 homolog (S. pombe)
NM_009009	Rad21	RAD21 homolog (S. pombe)
NM_009012	Rad50	RAD50 homolog (S. cerevisiae)
NM_011234	Rad51	RAD51 homolog (S. cerevisiae)
NM_011237	Rad9	RAD9 homolog (S. pombe)
NM_009391	Ran	RAN, member RAS oncogene family
NM_011249	Rbl1	Retinoblastoma-like 1 (p107)
NM_011250	Rbl2	Retinoblastoma-like 2
NM_133955	Rhou	Ras homolog gene family, member U
NM_144907	Sesn2	Sestrin 2
NM_030261	Sesn3	Sestrin 3
NM_018754	Sfn	Stratifin
NM_011368	Shc1	Src homology 2 domain-containing transforming protein C1
NM_013787	Skp2	S-phase kinase-associated protein 2 (p45)
NM_011407	Slfn1	Schlafen 1
NM_019710	Smc1l1	SMC (structural maintenance of chromosomes 1)-like 1 (S. cerevisiae)
NM_008017	Smc2l1	SMC2 structural maintenance of chromosomes 2-like 1 (yeast)
NM_009282	Stag1	Stromal antigen 1
NM_021465	Stag2	Stromal antigen 2
NM_009460	Sumo1	SMT3 suppressor of mif two 3 homolog 1 (yeast)
NM_020024	Taf10	TAF10 RNA polymerase II, TATA box binding protein (TBP)-associated factor
NM_009352	Terf1	Telomeric repeat binding factor 1
NM_009361	Tfdp1	Transcription factor Dp 1
NM_134138	Tnfsf5ip1	Tumor necrosis factor superfamily, member 5-induced protein 1
NM_011640	Trp53	Transformation related protein 53
NM_011641	Trp63	Transformation related protein 63
NM_021884	Tsg101	Tumor susceptibility gene 101
NM_009516	Wee1	Wee 1 homolog (S. pombe)

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TOXICOLOGICAL SCIENCES **103(1)**, 57–67 (2008)
doi:10.1093/toxsci/kfn021
Advance Access publication February 14, 2008

Genistein is an Efficient Estrogen in the Whole-Body throughout Mouse Development

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Received November 15, 2007; accepted January 2, 2008

The widespread use of diets containing estrogenic compounds raises questions on how relevant the presence of phytoestrogens may be, in order to allow a correct development of the reproductive ability and sexual maturity in humans and animals. The isoflavone genistein is the most estrogenically active molecule present in soy. Here we show that genistein, through an estrogen receptor (ER)–mediated action, modulates gene expression in the whole body of male mice in a dose- and time-dependent manner, at all ages. By luciferase bioassays, we show that genistein-induced ER activation is present in reproductive and nonreproductive organs of the transgenic mice Estrogen Responsive Element (ERE)-tK-LUC, although to an extent that is lower than what observed with the administration of estradiol. Peak activity was registered at genistein doses of 500–5000 μg/kg, at 12 h from the administration by gavage. In the liver, ER-α and ER-β messenger RNAs and two target genes, CYP17 and the progesterone receptor, were modulated by genistein. CYP17 and PR time-dependent induction was similar to that of luciferase. ER-α protein level followed an opposite regulation by genistein and estradiol. Genistein passed from the lactating mother to the suckling offspring at levels sufficient to activate gene expression in reproductive and non-reproductive tissues of the pups, with maximal upregulation at 16–24 h. We also followed responsiveness to genistein in the testis, from early development to adult age. Testis are well responsive to genistein as well as to estradiol already at day 14.5 of fetal development, as determined by exposing organotypic cultures from mouse fetus testis. Ovaries were not responsive under the same conditions. Activation of luciferase correlates with an activation of cell proliferation in testis, but not in the ovaries. Prolonged exposure (15 days) to genistein also decreases prostate weight like estradiol. In conclusion, our results show that genistein affects reproductive and nonreproductive organs of male mice in a dose- and time-dependent manner, at all developmental ages.

Key Words: **phytoestrogens; estrogen receptors; estrogen responsive elements; reporter mice; reproduction.**

It is now accepted that estrogens play a crucial role in the development and function of the male reproductive system (Couse *et al*., 1999, 2001; O'Donnell *et al*., 2001; Hess, 2003), as evidenced by the phenotype of estrogen receptors knockout mice (Eddy *et al*., 1996; Korach, 2000; Krege *et al*., 1998) and aromatase knockout mice (Robertson *et al*., 2002). The male reproductive tract—and probably the testis themselves—are responsive to estrogens from fetal life through adulthood, because they express estrogen receptors (ER-α and ER-β) and/or estrogen-related receptors from early stages of embryonal development (Nielsen *et al*., 2000). Recent studies indicate that in the mouse embryo, even the primordial germ cells, the precursors of oocytes and spermatogonia, and the somatic cells of the sex indifferentiated gonadal ridges, express ER-β or ER-α, respectively (Moe-Behrens *et al*., 2003; Mitsunaga *et al*., 2004). Thus, any compound that interacts with these receptors is potentially able to modulate and eventually interfere with the development of the reproductive system.

There are several chemicals produced by plants, present as natural constituent of food, that are able to bind to ERs (Cornwell *et al*., 2004). These compounds are called phytoestrogens and are present at high levels in certain foods such as soy. Although a great amount of research on the toxicological, nutritional, and pharmacological properties of phytoestrogens has been conducted, a sufficiently informative picture of their targets and biological effects is still unavailable.

People are exposed to these nutritional estrogens depending on the type and daily intake of vegetables. Asian populations may assume from 30 to 50 mg/day of isoflavones from soy (Adlercreutz *et al*., 1993; Kimira *et al*., 1998) equivalent to about 1 mg/kg/day of total genistein. These intakes produce blood levels of isoflavones ranging from 100 to 500 nanomolar (nM) (Setchell *et al*., 1998; Uehar *et al*., 2000). Because in previous experiments on mice we have found that blood levels of genistein in this concentration range exert estrogenic effects (Penza *et al*., 2006, 2007), it can be postulated that these dietary levels of genistein can reach estrogenically active levels also in humans. Finally, it must be taken into consideration that

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human infants drinking soy-based formulas are exposed to much higher levels of genistein than adults on soy-containing diets. Their blood levels may reach 1–5μM total genistein, ten times higher than the amount found in adults' serum. Moreover, the infants show a much higher level of genistein aglycone, which is the active form of genistein (Chang *et al*., 2000; Doerge *et al*., 2002).

A further concern arises from the knowledge that both ER-α and ER-β receptors or ER-β only depending on the cell type, are expressed in human ovary and testis, respectively, during fetal life (Gaskell *et al*., 2003; Vaskivuo *et al*., 2005). Very controversial are, however, the studies reported on the effects of phytoestrogens in male, particularly for their effect on development and the reproductive health following perinatal exposure. In the offspring, phytoestrogens might potentially affect both the germ and somatic testicular cells with consequences involving a complex array of pathological changes, collectively known as the testicular dysgenesis syndrome (for a review see Sharpe, 2001).

At present, there are some questions about the estrogenic effects of isoflavones that still need to be clarified. First, it is yet not clear whether the estrogenic effect observed with genistein on the whole physiology and in particular on the reproductive tract are mainly due to the activation of ER-mediated pathways. Second, it is yet not clear what are the active estrogenic concentrations of genistein, because in few studies genistein effects were observed only at pharmacological concentrations (Nagao *et al*., 2001). Third, what is the most sensitive period of life where exogenous estrogens may alter tissue-specific gene expression profiles and development.

In this work we have analyzed, by a whole-body approach, the estrogenic dose-dependent effect of genistein on several tissues of adult estrogen reporter transgenic male mice engineered to express a reporter of ER transcriptional activity (ERE-tK-LUC mouse), a model that provides a functional picture of the map of ER activation (Ciana *et al*., 2001, 2003; Maggi *et al*., 2004; Penza *et al*., 2004; Villa *et al*., 2004). The doses of genistein used were representative of the intake typical of western diets (low intake, from 5 to 50 μg/kg/day) and eastern diets (high intake, from 50 to 500–1000 μg/kg/day), and the intake of infants fed with soy-based formulas and soy-based nutritional supplements (high intake, from 500 to 5000 μg/kg/day) (Setchell *et al*., 1997). We used this mouse model also to analyze the effect of genistein exposure of lactating mothers on suckling estrogen reporter pups. Finally, we used *in vitro* culture system to investigate whether genistein exerts estrogenic action on the gonads of fetus, adults, and developing mice.

MATERIALS AND METHODS

Experimental animals. The procedures involving animals and their care were conducted in accord with institutional guidelines, which comply with national and international laws and policies (National Institutes of Health, Guide for the Care and Use of Laboratory Animals, 1996 (7th ed.) [Washington, D.C.]; National Academy Press, National Research Council Guide, www.nap.edu/readingroom/books/labrats). ERE-tK-LUC transgenic mice were kept in animal rooms maintained at a temperature of 23°C, with natural light/dark cycles. For the experiments, we used heterozygous littermates, obtained by mating our founders with C57BL/6J wild-type mice. Heterozygous transgenic male mice were screened by PCR analysis for the presence of the transgenic cluster. Before treatments, heterozygous male mice (2 months old) were put 4 days on an estrogen-free diet (Piccioni, Italy). Genistein was given by oral gavage, which is its normal route of assumption, estradiol by ip injection. Vegetal oil was used as vehicle and negative control. The animals were sacrificed by cervical dislocation and the tissues dissected and immediately frozen on dry ice. Tissue extracts were prepared by homogenization in 500 μl of 100mM K_2PO_4 lysis buffer (pH 7.8) containing 1mM dithiothreitol, 4mM ethylene glycol tetraacetic acid, 4mM ethylenediaminetetracacetic acid (EDTA), and 0.7mM phenylmethylsulfonylfluoride, with three cycles of freezing–thawing and 30 min of microfuge centrifugation at maximum speed. Supernatants, containing luciferase, were collected, and protein concentration was determined by Bradford's assay (Bradford, 1976).

We also analyzed the effect of genistein exposure of lactating mothers on suckling estrogen reporter pups at 4 days of age. Eventually, we investigated whether genistein exerts estrogenic action on fetal testis of the transgenic mice. Fetal testis were then dissected from embryos obtained by breeding ERE-tK-LUC with C57BL/6J or where indicated from CD-1 mice. The day of plug was considered as 0.5 days postcoitum (dpc).

Chemicals. Estradiol (17β-E2) and genistein were purchased from Sigma (Pomezia, Italy); β-benzene-hexachloride was purchased from Superchrom (Milan, Italy).

Enzymatic assay. Luciferase enzymatic activity was measured, as reported by De Vet *et al*. (1987), in tissue extracts at a protein concentration of 1 mg/ml. The light intensity was measured with a luminometer (Digene Diagnostics, Gathersburg, USA) over 10 s and expressed as relative light units/mg proteins.

Protein isolation and Western analysis. Tissues were excised and homogenized in lysis buffer (1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [pH 7.9]; 2M $MgCl_2$; 10% [vol/vol] glycerol; 5M NaCl; 0.5M EDTA; 0.1% Triton-X 100; 14.2M mercaptoethanol; 100mM phenylmethanesulphonylfluoride) supplemented with a protease inhibitor cocktail. Homogenized was centrifuged for 25 min at 13209 in cold room. Protein concentration was determined by the Bradford's assay. Equal amount of proteins from each treatment group were boiled in 1× Laemmli buffer (50mM Tris–HCl, 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, 175mM β-mercaptoethanol). Proteins were separated by SDS–10% polyacrylamide gel electrophoresis, and electrophoresed to polyvinylidene difluoride membrane. Membranes were blocked in Blotto (5% milk, Tris-buffered saline (10mM Tris–HCl, pH 8.0, 150mM NaCl), and 0.05% Tween 20) and probed with primary antibodies ER-α (R21) (1:1000) and ER-β (CO1531) (antibodies were a gift from J. Greene, Chicago, IL). Following incubation with peroxidase-conjugated secondary antibody, immunoglobulins were visualized using the enhanced chemiluminescence detection system (Pierce, Milan, Italy). Densitometric quantitation of ER-α levels relative to actin levels was performed using the Matrix software program (Quantavision, Canada).

Messenger RNA quantification by real-time reverse transcription–PCR. Total RNA was extracted from 10 to 30 mg of tissue using the Nucleospin RNA II kit (BD Bioscience, San Josè, CA) and following the manufacture's instruction. RNA for each sample was reverse transcribed using the High capacity complementary DNA Archive Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed using the Assay on Demand kit based on TaqMan chemistry (Applied Biosystems). Reverse transcription–PCR (RT-PCR) reactions were performed on an ABI PRISM 7000 Sequence Detection System instrument and data analysis was done with the ABI PRISM

7000 SDS software (Applied Biosystems). 18S RNA was used as referent for housekeeping gene. Specific oligonucleotides pair were designed by the Applied Biosystems service. Calculations were done as described for the Comparative Method in the User Bulletin 2 of ABI PRISM sequence detection system.

Gonad culture. Testis and ovaries obtained from 14.5 dpc embryos were cultured in 0.5 ml of RPMI medium free of phenol red plus 2% charcoal-stripped fetal calf serum in a 1.5-ml Eppendorf tube. For luciferase assay, gonads from ERE-tK-LUC embryos were maintained in medium containing the compound to be tested (see "Results") for 6 h at 37°C and 10% CO_2 in air. Tissues were then harvested and homogenized in TEN buffer (10mM Tris [pH 8.0], 150mM NaCl, 10mM EDTA). Cell pellets were lysed in luciferase assay buffer (25mM Tris [pH 8.0], 150mM NaCl, 10mM EDTA, 1mM dithiothreitol, 5% glycerol, 0.5% Triton-X 100) and passed through a 26-gauge needle 20 times. The lysate was then spun for 20 s at 21,000 × g, and the supernatant assayed for luciferase activity. For ^{3}H-thymidine assay, gonads from CD-1 mice were cultured for 24 h in medium containing the compound to be tested and for the last 4 h 2.5 μCi/ml ^{3}H-thymidine. Gonads were extensively washed, transferred in microtiter plates and the count per minute measured with a Top Count β/γ counter (Canberra Packard Instruments Company, Meriden, Connecticut, 06450, USA).

Statistical analysis. Statistical analysis was performed by two-way analysis of variance test followed by *post hoc* Bonferroni analysis.

RESULTS

Map of ER Activation by Genistein in Mouse Organs

The ERE-mediated transcriptional activity of genistein was tested *in vivo* in the transgenic ERE-tK-LUC reporter mouse line. A dose response analysis was determined in several tissues of male mice orally treated with 0, 5, 50, 500, 5000 μg/kg of the compound. These amount of genistein gave a serum concentration respectively of 30 ± 4; 35 ± 6; 77 ± 8; 86 ± 8; 92 ± 9nM 12 h after the gavage.

The response was quantified by assaying the enzymatic activity of the transgenic marker luciferase (LUC). In parallel, mice were also treated with estradiol as a reference compound. As reported in Figure 1 genistein showed the ability to modulate the ERs, although at different concentrations and in a tissue preferential manner. Maximal increase in ER action was almost always evident at a concentration between 500 and 5000 μg/kg (Fig. 1) (500 μg/kg are equivalent to the intake of 30 mg for a 60 kg person). Liver and thymus responded to the compound already at a concentration of 50 μg/kg. Genistein was a less efficient inducer in the pancreas, eye, and cerebellum. Peak activity was registered at 12 h and started to decrease at 24 h in all the tissues being more evident in the liver (Fig. 2).

We did not notice any differential effect in ERs activation in tissues expressing different levels of the ERs, as the liver versus the lung. The response to genistein never approached that of estradiol and is delayed with respect to the natural estrogen, which peaks at 6 h. Measurement of luciferase in tissues of animals treated with vehicle alone showed a stable basal activity of the transgenic marker.

Reproductive tissues were also weighed after 15 days of exposure. We noticed a significant decrease in seminal vesicles and testis weight of mice exposed 6 days after weaning (4 weeks old) to estradiol but not genistein. The prostate weight significantly decreases with both compounds. Body weight was unaffected by genistein, whereas it significantly decreased with estradiol. The effect of genistein was dose dependent and inversely correlated with the reporter upregulation (Figs. 3A–D).

Comparison between the Regulation of Endogenous Genes and Luciferase

To verify if luciferase induction reflected modulation of estrogen-dependent gene expression by genistein, we determined in the liver of genistein exposed mice the level of the progesterone receptor (PR) and the steroidogenic P450c17alpha (CYP17) messenger RNAs (mRNAs), known targets of the estrogens in this organ. PR and CYP17 mRNAs were quantified by real-time RT-PCR in mice treated for 1, 3, 6, 12, 24 h with 500 μg/kg genistein or 5 μg/kg estradiol. As shown in Figure 4, PR and CYP17 mRNAs induction was maximal at 6–12 h, a kinetics that appear a little early, compared with luciferase upregulation, which is maximal at 12–24 h. Both genes were also upregulated by estradiol (Fig. 4A).

The modulation of ER-α and ER-β mRNAs by genistein and estradiol in the liver was also evaluated. ER-α and ER-β mRNAs were upregulated by genistein following a time course shorter than that of luciferase (maximal level at 3 h). In estradiol treated mice, however, only the ER-β mRNA was upregulated. Interestingly ER-α protein level was downregulated in the liver by genistein (Fig. 4B) indicating that the regulation of transcription or mRNA synthesis/stabilization does not follow the same trend of the ligand-dependent protein turnover, possibly due to a regulatory effect on the proteasome degradation pathway (Marsaud *et al*., 2003). ER-β protein was at undetectable levels (not shown). ER proteins are modulated by genistein also in the testis and lung of 2 months old mice further confirming that ERs modulation by nutritional doses of genistein is an effect occurring in different body systems. In the testis ER-α modulation inversely correlates with the induction of luciferase being ER-α maximally downregulated at the time and doses where induction of luciferase was at the highest levels (500 μg/kg; 6–12 h) (Fig. 4C). A strong decrease of ER-β occurred in testis at 24 h, whereas in the lung it was maximal already at 3 h, respectively later and much earlier than luciferase increase in the same organs.

Activity of ER in 4-Day-Old Pups Nursed by Genistein Treated Mothers

Because exposure to phytoestrogens through soy-containing infant formulas has been hypothesized to be harmful for development of the male reproductive system and adult fertility

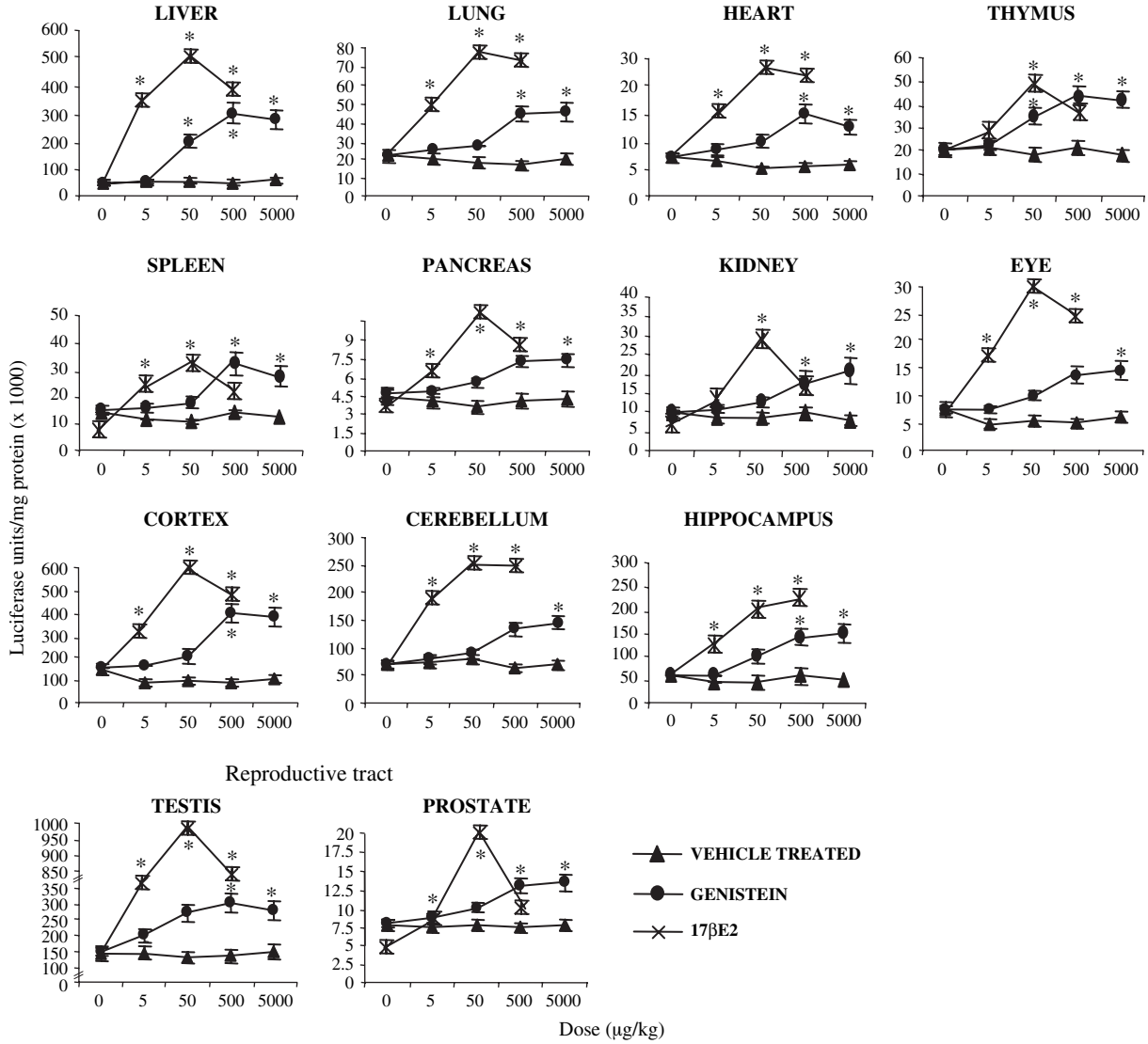

FIG. 1. Effects of different oral doses of genistein and estradiol in ERE-tK-LUC male mice. Luciferase activity in tissue extracts was expressed as relative light units normalized on protein concentration. Two-month-old male mice were put on an estrogen-free diet for 1 week before treatments. Mice were treated with a single dose of 0, 5, 50, 500, 5000 μg/kg genistein by oral gavage or a single dose of 0, 5, 50, 500 μg/kg estradiol and sacrificed 12 h later. The experiments were repeated three times, with a total of 12 animals per group. Values are plotted on a semilogarithmic scale. At the two highest concentrations of compounds tested (500 and 5000 μg/kg) all the values reported for liver, thymus, lung, heart, spleen, testis, hippocampus, and cortex are significantly different from controls (*$p < 0.05$). Results in the pancreas, kidney, eye, and cerebellum are significantly different than controls at the dose of 5000 μg/kg (*$p < 0.05$). In the liver and the thymus significant induction was reached already with 50 μg/kg genistein. Estradiol significantly induced luciferase almost at all the doses used, although maximal activation was observed at 50 μg/kg. Results are expressed as means of 12 determination for each treatment group. Bars represent the average ± SEM. *$p < 0.05$ as compared with the relative controls.

(Chen and Rogan, 2004), we used ERE-tK-LUC pups to investigate whether genistein exerts estrogenic action in breast-fed pups fed by genistein exposed mothers. Lactating mothers were given a single dose of genistein (50 mg/kg) by oral gavage at day 4 after delivery. This amount produced in the dam a serum genistein level of 550 ± 109nM (not shown). ERE-tK-LUC suckling pups were killed at 0, 6, 16, and 24 h after the mothers' treatment and luciferase activity was measured in the liver, lung, hearth, thymus, testis, and brain (Fig. 5). Mothers' treatment resulted in an increased luciferase activity in all pup organs examined, which resemble the expression observed in the adults, although it appears more consistent in the lung and testis. This indicates that genistein passes from the mother to the milk at concentrations sufficient to exert estrogenic actions on reproductive and nonreproductive tissues of breast-fed newborns.

Estrogenic and Proliferation Action of Genistein on Fetal Testis

We then investigated whether genistein can exert estrogenic action directly on fetal gonads.

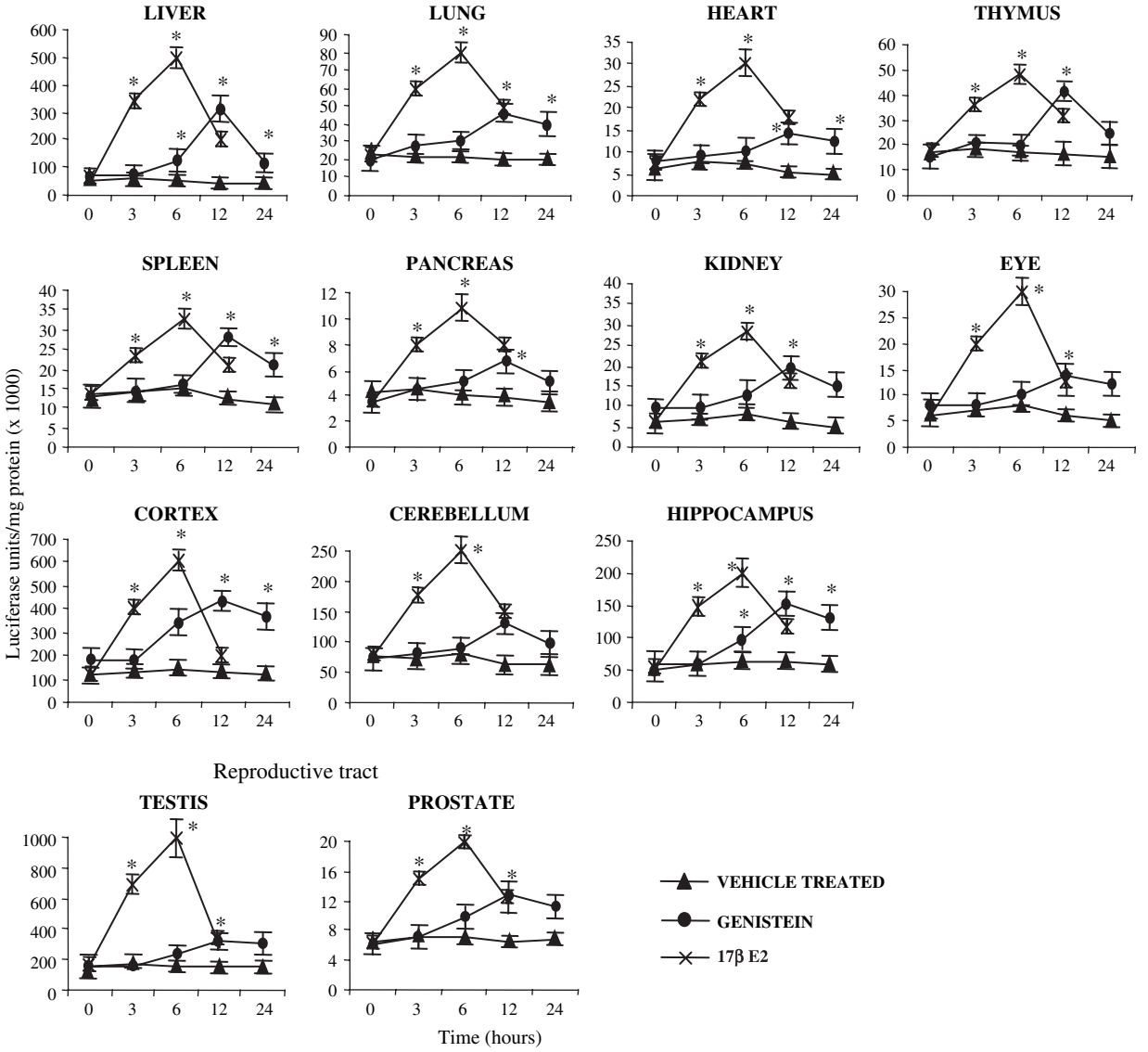

FIG. 2. Time-dependent regulation of the estrogen driven reporter after a single oral dose of genistein or estradiol in ERE-tK-LUC male mice. Two-month-old male mice were put on an estrogen-free diet for 1 week before treatments. Vehicle (vegetal oil) or an oil solution of 50 μg/kg of estradiol (ip) or 5000 μg/kg of genistein by oral gavage were given. Mice were sacrificed after 0, 3, 6, 12, 24 h. Serum and tissues were collected and stored at −80°C until assayed. Luciferase activity in tissue extracts was expressed as relative light units normalized on protein concentration. The experiments were repeated three times, with a total of 10 animals per group. Results are expressed as means of 10 determination for each treatment group. Values are plotted on a semilogarithmic scale. Bars represent the average ± SEM. *$p < 0.05$ as compared with the relative controls.

To this aim, testis and ovaries of 14.5 dpc mouse embryos were chosen because at this age there is evidence for expression of both ER mRNAs and proteins in the developing testis and ovary (Greco *et al.*, 1991, 1993; Jefferson *et al.*, 2000; Lemmen *et al.*, 1999; Nielsen *et al.*, 2000).

Gonads were dissected from 14.5 dpc ERE-tK-LUC embryos and cultured in the presence of 1μM genistein, 10nM estradiol or vehicle (ethanol) for 6 h at 37°C, before running the luciferase assay. Figure 6A shows that luciferase was induced twofold in testis exposed to genistein or estradiol and the addition of the antiestrogen ICI-182780 inhibited this response. Ovaries did not show any significant response to either genistein or estradiol (Fig. 6B).

We then tested whether genistein and estradiol were able to affect the proliferation of testicular and ovarian cells using a ^{3}H-thymidine incorporation assay. Both compounds appeared to stimulate testicular cell proliferation as revealed by a significant twofold increase of ^{3}H-thymidine incorporation in cultured testis, which is inhibited by the antiestrogen ICI-182780 (Fig. 7A). Ovaries were unresponsive to the same treatments (Fig. 7B).

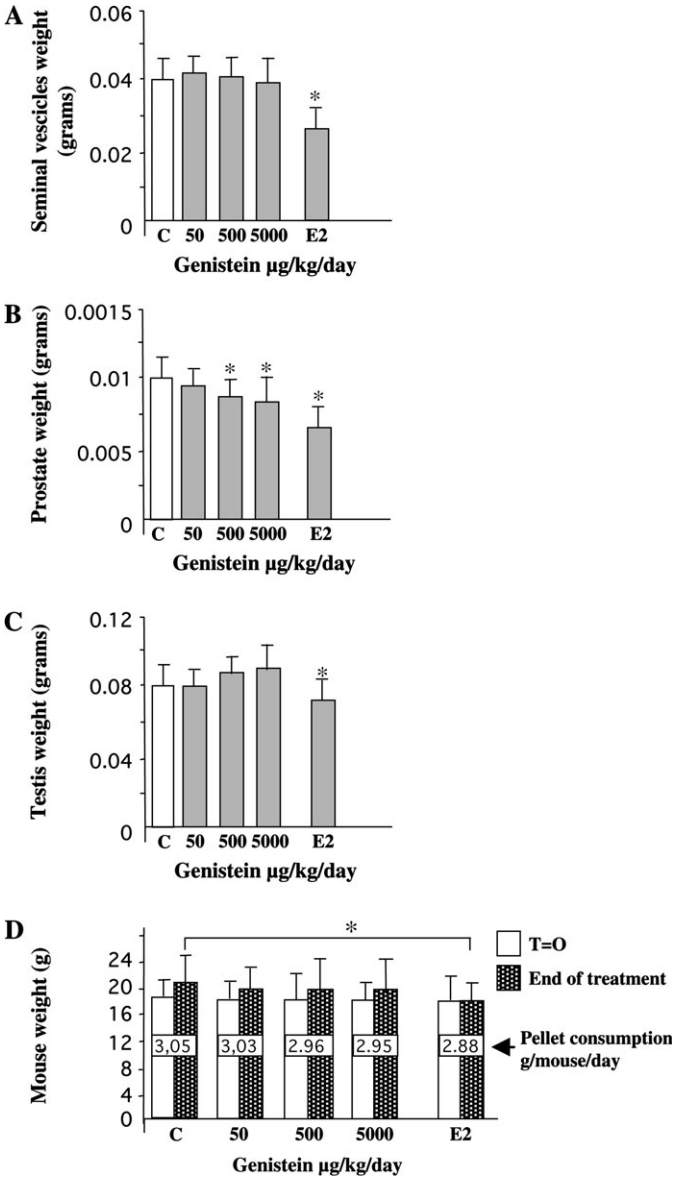

FIG. 3. Reproductive organs weight in genistein treated mice. (A, B, and C) Four-week-old mice were put for 15 days on a soy-free standard diet. Mice received genistein by oral garage at doses of 0, 50, 50, 5000 µg/kg or estradiol at the dose of 5 µg/kg. Seminal vesicles, prostate, and testis were excised and weighed (y-axis; grams). Control group: vegetable oil. Treatments were given once a day for 15 days. (D) The total body weights (grams) and food intake (grams per mouse per day) are shown. Measurements were made daily at 5:00 P.M.. Bars indicate the average weight for each group of mice at the beginning ($T = 0$) and before sacrifice (end of treatment). Average pellet consumption is indicated in the histograms. Data are mean ± SEM. *$p < 0.05$ versus controls.

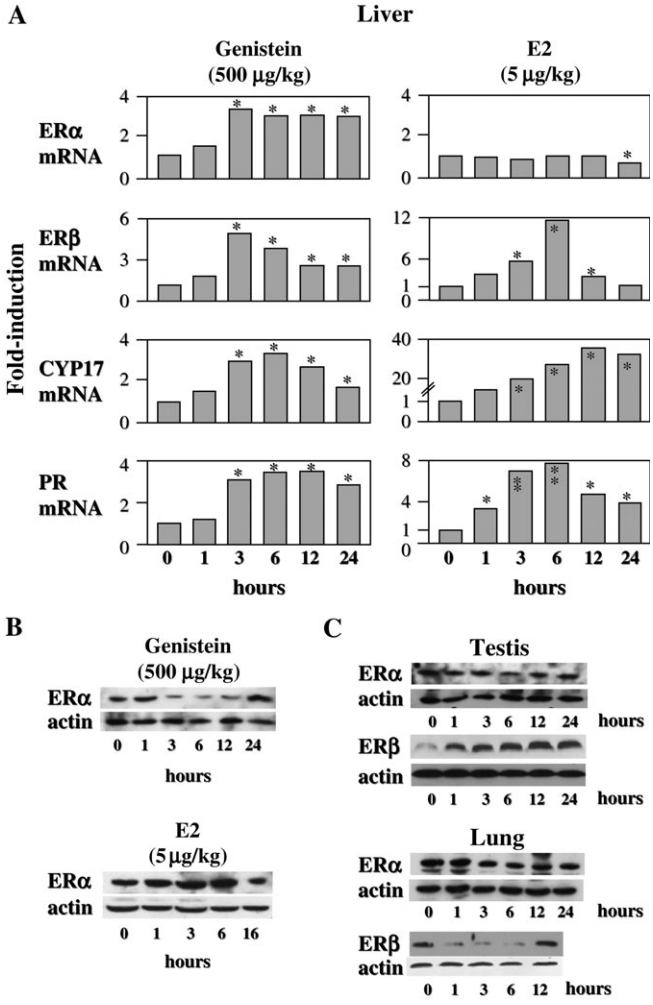

FIG. 4. Effect of genistein and estradiol on the expression of ER-α, ER-β, and target genes. (A) The data show the relative levels of PR, CYP17, ER-α, and ER-β mRNAs in liver of mice acutely treated with 500 µg/kg genistein or 5 µg/kg estradiol for 1, 3, 6, 12, 24 h (five mice per group). The expression is shown relative to the ribosomal 18S RNA. (B) Western blot analysis of ER-α in the liver. Mice were treated as above. Liver extracts from the different treatment groups were analyzed by Western blot analysis with anti-ER-α antibodies (H222) and anti-β-actin antibodies. (C) Western blot analysis of ER-α and ER-β in testis and lung. Mice were treated 500 µg/kg of genistein by oral gavage for different lengths of time (0, 1, 3, 6, 12, 24 h). Tissues extracts from the different treatment groups were analyzed by western blot analysis with anti-ER-α (H222), anti-ER-β (CO1531) and anti-β-actin antibodies. Data in (A) are shown as means ± SEM for each group. Values with asterisks are significantly different from control (*$p < 0.05$; **$p < 0.001$).

DISCUSSION

We used the ERE-tK-LUC transgenic mice as a model to test the hypothesis that genistein, at doses found in diets containing various amount of isoflavones, has estrogenic effects on the whole body, including reproductive organs.

We show here that genistein doses, as present in a single meal containing moderate to high amounts of soy, or in the assumption of a common isoflavone-containing food supplement (30 mg, equal to 500 µg/kg), produce in many tissues (liver, heart, thymus, spleen, testis, cortex) a response that is equal or very close to the maximal response observed with the 5000 µg/kg dose. The peak in reporter induction in adult mice was detectable at 12 h and is compatible with the time-dependent profile of

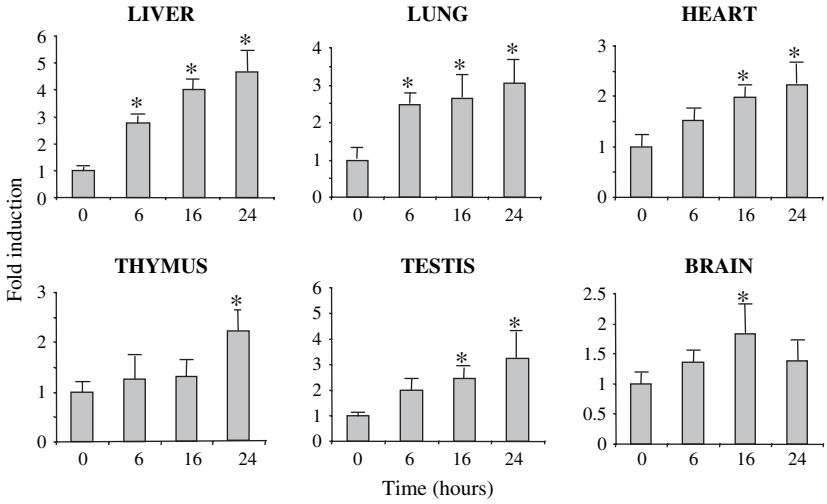

FIG. 5. Estrogenic action of genistein in tissues of 4-day-old suckling estrogen reporter pups of genistein treated mothers. Lactating mothers were treated by oral gavage with a single bolus of 50 mg/kg body weight of genistein. Controls were treated with vegetal oil. Luciferase activity was measured in the liver, lung, heart, thymus, testis, and brain at 0, 6, 16, 24 h after mother treatment. Bars represent the average ± SEM of three individual experiments, each performed in duplicate. *$p < 0.05$, as compared with the control.

genistein seen in the plasma of human subjects after assumption of a single dose of genistein (King and Bursill, 1998). Reporter activation reflected, although with a slightly different kinetic, the regulated expression of target endogenous genes (CYP17, PR, ER-α, and ER-β). The action of genistein was always less potent than estradiol, except for the PR gene that was induced almost at the same level.

ER levels are modulated by genistein in the liver, testis, and other organs such as the lung, proving that their involvement/recruitment in genistein action occurs in the whole body. ERs modulation by genistein was observed to be tissue specific. In the liver genistein was efficient in inducing ER-α mRNA, and downregulating the ER-α protein, an effect not elicited by estradiol, which showed an opposite action on ER-α protein. Similarly to estradiol, genistein induced upregulation of ER-β mRNA. Previous reports (Shupnik *et al.*, 1998) showed that a ligand induces upregulation of ER-α protein, that occurs in the liver, but not in other tissues. Here we confirm this finding, but we also show that it is specific for the endogenous ligand, because genistein always downregulated ER-α protein. In this and other organs, on the contrary, genistein upregulated ER-α mRNA, whereas estradiol caused a significant mRNA downregulation.

It is known that the ER levels may determine the cellular response to ER ligands (Meegan and Lloyd, 2003). On the other hand ER stability is influenced by specific ligands (Wijayaratne and McDonnell, 2001), which may condition the interaction of the receptor with tissue-specific coregulators

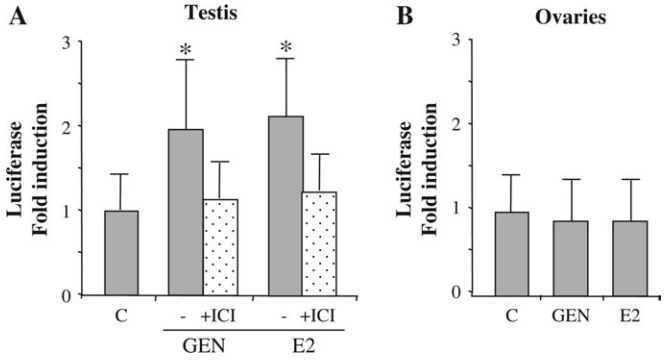

FIG. 6. Estrogenic action of genistein on fetal gonads. At 14.5 dpc testis (A) and ovaries (B) obtained from estrogen reporter embryos were exposed to 1μM genistein, 10nM estradiol, or vehicle (ethanol), in culture for 6 h and subjected to the luciferase assay as reported in ''Materials and Methods.'' Bars represent the average ± SEM of two individual experiments, each performed with at least three gonads per group. *$p < 0.05$, as compared with the control.

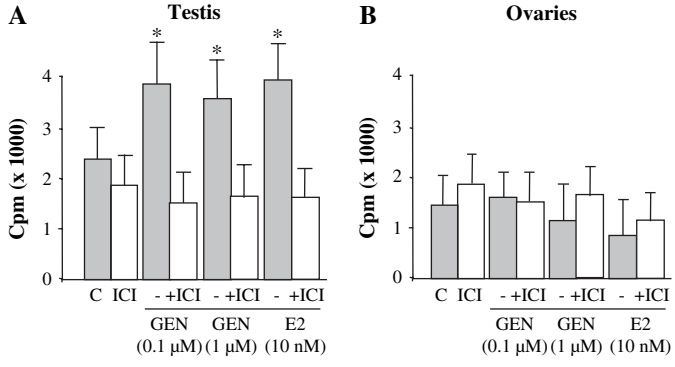

FIG. 7. Effect of genistein and estradiol on fetal gonadal cell proliferation. ^{3}H-thymidine incorporation in 14.5 dpc testis (A) and ovaries (B) obtained from CD-1 mice and cultured for 24 h in the presence of 0.1 and 1μM genistein, 10nM estradiol, or vehicle (ethanol). The experiments were repeated twice, with a total of at least six gonads per group. Bars represent the average ± SEM of two individual experiments, each performed in triplicate. *$p < 0.05$, as compared with the control.

(Nilsson *et al*., 2001) and ER-associated proteins (Saceda *et al*., 1998) or with the proteasome degradation pathway (Marsaud *et al*., 2003). Lonard *et al*. (2004) reported that ER-α bound by selective etrogen receptor modulators 4-hydroxytamoxifen and raloxifene, increases the steady-state levels of ER-α protein itself and of ER-coactivators in a cell type-specific way, and that this event has functional consequences on the transcriptional activity of ERs.

We show here that although the modulation of ER-α, which is the only isoform detectable in the liver at protein level, differs in genistein and estradiol, the ERE-dependent activation of luciferase is similar and reflects what occurs in classical targets of estrogens, such as the PR and the steroidogenic enzyme CYP17. Besides the fact that this strengthens our data on the modulation of gene expression analyzed with reporter mice, it also indicates that genistein, although less potent than estradiol, may have functional consequences on estrogen synthesis and activity by strongly inducing the CYP17 gene and on the response of mouse tissues to progestinic compounds by upregulating the PR (Scippo *et al*., 2004).

Infants' daily intakes of phytoestrogens from human milk are calculated to be 5–10 μg/kg. This amount is very low if compared with the amount provided by soy-based infant formulas (6000–11,000 μg/kg) (Setchell *et al*., 1997, 1998). Thus, it seems that there is no reason for concern about the maternal–infant transfer of phytoestrogens from human breast milk during breast feeding. On the basis of the weak estrogenic activity of isoflavones it is unlikely that the dietary intake from human milk is sufficient to exert significant estrogenic effects. This may be different for diets rich in phytoestrogens, such as those of people consuming soy supplement or soy-containing diets. Several authors showed that suckling rats are exposed to active levels of genistein passed from the mother during lactation (Doerge *et al*., 2001; Fritz *et al*., 1998; Hughes *et al*., 2004). They showed by assaying genistein levels in the mother and in suckling pups sera, that lactational transfer of genistein occurs, although exposure is generally lower than that measured in the fetal period. Here we report for the first time data proving that nutritional doses of genistein given to the dam and that produced blood levels in the dam of 550 ± 109nM, passed to the pups during lactation in an amount sufficient to exert estrogenic actions in 4-day-old suckling mice. The six tissues analyzed, including the brain and reproductive organs, were all well responsive to genistein at 16–24 h after the mother treatment.

In other experiments Doerge *et al*. (2006) also showed that genistein crosses the rat placenta and can reach the fetus. Although fetal and neonatal serum concentration of total genistein were approximately 20-fold lower than maternal concentrations, the biologically active genistein aglycone concentration was only fivefold lower. Fetal brain contained predominately genistein aglycone at levels similar to those in the maternal brain, thus indicating that genistein can reach all tissues in the pups.

The effects of genistein on reproductive development and functions are still controversial. Several reports have shown that genistein exposure during gestation and lactation has no adverse effects on reproductive organs, fertility, and embryonic development (Fielden *et al*., 2002, 2003; Fritz *et al*., 2003; Jung *et al*., 2004; Kang *et al*., 2002; Lee *et al*., 2004; Roberts *et al*., 2000). Other studies, on the contrary, have reported adverse effects (Delclos *et al*., 2001; Lewis *et al*., 2003; Nagao *et al*., 2001; Strauss *et al*., 1998), at doses comparable to those administered in the present study. These effects consist in a decreased fertility and estrous cycle changes in female and male rats, although these effects were observed only at the pharmacological dose of 100 mg/kg/day (Nagao *et al*., 2001). Wisniewski (Wisniewski *et al*., 2003) also reported that perinatal exposure to genistein resulted in alterations in masculinization of the reproductive system in male rats, an effect probably due to the estrogenic action of genistein. In other studies, genistein acted on adult mice as an estrogen at nutritional doses, but it was effective only at pharmacological doses when mice were exposed during perinatal life (Strauss *et al*., 1998). In a more recent work Jung *et al*. (2004) postulated that the severe impairment observed in the male reproductive system of rats exposed to genistein during perinatal life, might be due to its estrogenic action. This was further supported by the work of Nam *et al*. (2003) in which the author, reported that the expression of genes target of estradiol were upregulated by the estradiol and tamoxifen in the testis and prostate of mice. Many tests have shown that genistein inhibits the induction of acrosomal exocytosis and binding of spermatozoa to the zona pellucida, without affecting sperm motility parameters (Hinsch *et al*., 2000). Fielden *et al*. (2003) and Jung *et al*. (2004) reported that exposure to genistein doses higher than those used in this study, significantly increased the *in vitro* fertilizing ability of epididymal sperm by increasing their motility.

Taken together, these different studies on the effects of genistein on reproduction produced conflictual results. The data reported in the present paper show that genistein at a dose that may be found in serum of people on soy-containing diets (1μM) is estrogenic in reproductive organs of adults mice and in the male fetus, at the gestational age of 14.5 days. The testis, but not the ovaries responded to genistein as well as to estradiol, by upregulating the reporter twofold through an ER-mediated mechanism. This effect was accompanied by a testicular increase in cell proliferation which was also ER mediated. It is not surprising that only the testis responded to estrogens at the developmental age of 14.5 dpc because in the mouse ovaries there is no detectable expression of ERs, whereas both ER-α and ER-β are expressed in the testis (Delbès *et al*., 2004; Greco *et al*., 1993; Jefferson *et al*., 2000).

Sensitivity to natural and synthetic estrogens varies among species and strains (Long *et al*., 2000). In this regard, it is interesting to note that significant estrogenic effects of genistein on cell proliferation resulted from exposure of 14.5-day-old

mouse testis of CD-1 mice, a strain which is among the least sensitive to natural estrogens (Spearow *et al*., 2001).

The observed effect on cell proliferation, in addition to the modulation of the ERE-dependent luciferase, suggests that prenatal exposure to genistein may directly alter developmental process of male gonads, possibly compromising male fertility later in life. Although indirect effects have also been reported on the testis through the modification of the function of the hypothalamic-pituitary-gonadal axis (Hilakivi-Clarke *et al*., 1999).

Models of the molecular activity of estrogens in mouse tissues have revealed new and unexpected roles for ER ligands, such as genistein. These models, beyond the ones of targeted gene disruption such as ER-α and ER-β knockouts (Eddy *et al*., 1996; Korach, 2000; Krege *et al*., 1998) or ER-α/ER-β double knockouts (Islander *et al*., 2003), include estrogen reporter mice (ERE-tK-LUC) (Ciana *et al*., 2001). We have shown here and in recent works (Penza *et al*., 2006, 2007) that ERE-tK-LUC mice represent a suitable model for assessing the estrogenicity of genistein in the whole body and its activity in a tissue dependent way, during development.

Our results may highlight an important effect of genistein in reproductive and nonreproductive organs of male mice. The effect of this phytoestrogen may differ depending on the dose administered and on the age at the time of administration, although at all age mice seem to be able to respond to this compound. Further examination of the effects of genistein should strengthen our understanding of the etiology of the incidence of reproductive diseases in humans on diets rich in phytoestrogens. In turn, this should help nutritionists to determine more accurately the benefit or the risk of estrogen containing diets during pregnancy and development.

FUNDING

European Union grants (QLK4-CT-2002-02221) (EDERA), (LSHB-CT-2006-037168) (EXERA); Istituto Superiore di Sanità (ARACNA); and Ministero dell'Università e della Ricerca Scientifica; Fondazione Cariplo, Bando 2006.

ACKNOWLEDGMENTS

We are indebted to Geoffrey Greene for the gift of anti-ER-α and anti-ER-β antibodies and to Francesca Piazza and Alessandro Bulla for their helpful English writing and editing assistance.

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