UNIVERSITÀ DEGLI STUDI DI MILANO

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Corso di Dottorato in Scienze Farmacotossicologiche, Farmacognostiche e Biotecnologie Farmacologiche

XXIII CICLO

Settore Scientifico Disciplinare BIO/14

"FLUCTUATION OF ESTROGEN RECEPTOR TRANSCRIPTIONAL ACTIVITY IN PHYSIOLOGICAL CONDITIONS: FUNCTIONAL CONSEQUENCES"

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Anno Accademico 2009/2010

1. Estrogens and their receptors

Estrogens belong to the wide family of steroid hormones, and function as the primary compounds in the regulation of the female reproductive activity, despite it appears to play an important role even in the male physiology. Their name comes indeed from a Latin word "estrus/oistros" that indicates a period of fertility for mammals. 17-β-estradiol (E2), estrone (E1) and estriol (E3) are the main naturally occurring estrogenic compounds in the female organism; during the reproductive years, estradiol is the predominant form, and is produced by the granulosa cells of the ovaries, even if secondary sources of estrogens are liver, adrenal glands, fat cells, breast. Estrone is produced during menopause, and estriol is the primary estrogen of pregnancy.

Their lipophilic structure allows them to permeate across the biological membranes of cells, constituted by a double phospholipidic bilayer, and to diffuse in the cytoplasm; once inside the cells the hormones can bind the specific intracellular receptor, and trigger the cellular response, that eventually implies the modulation of gene expression.

During the reproductive cycle in the sexually mature female, luteinizing hormone (LH) secreted by the anterior pituitary gland stimulates the production of estrogen in the ovaries during the first phase of the ovulation, named follicular phase. Estrogen synthesis and secretion in the bloodstream reach the peak before the middle of the cycle, when the progesterone production begins, triggering the ovulation. After the ovulation, estrogens and progesterone are poorly synthesized from granulose cells of corpus luteum for the remaining days of the cycle.

With the menopause, ceasing the ovaries gametogenic and endocrine function, estrogens are no longer produced. Notwithstanding, some women show significant plasmatic hormone levels, thanks to the increased transformation of surrenalic steroids, such as androstenedione, to estrone and estradiol in the adipose tissue and in other non endocrine tissues.

Once freed in the bloodstream, estrogen, that is indeed a hydrophobic molecule, binds with high affinity to a steroid hormone binding β2-globulin (SHBG), and with lower

affinity to albumin. The bound hormone is prevented from the diffusion across the membranes, therefore only the free fraction is available for diffusion and represent the physiological active form. Thus bioavailability of sex hormones is influenced by the level of SHBG.

As said above, estrogen once inside the cells bind their natural receptor, called indeed Estrogen Receptor (ER). Estrogen receptor is present in two different isoforms: the α isoform, that is the first identified and is localized on chromosome 6, and the β isoform, identified in 1986 and localized on chromosome 14. The sequence homology between ERα e ERβ reach 96% in the DNA binding domain, but as low as 53% in the ligand binding domain; this would suggest that ERβ recognizes with the same affinity the genomic sequences bound by ERα, but that is characterized by a different array of ligands. Even the distribution of the two isoforms in the body tissues is different: a number of different studies showed that the estrogenic action is mediated by both isoforms in some central nervous system areas, in the cardiovascular system, in the uterus, in the mammary gland; α isoform prevails in liver, in testis and epididimus, while β isoform is more represented in prostate, lung, intestine, bone, kidney, midollar bulb and spine cord.

2. Estrogen Receptors

Signaling molecules, or ligands, are chemicals involved in the transmission of information between cells, and work thanks to their ability to selectively bind to specific receptors after being released from the cell sending the signal. There are in fact many different kind of receptors, and each kind of receptor can bind only certain ligand shapes. When the signaling molecule interacts with the receptor, a ligand-induced change in receptor conformation and activity is established, triggering a cellular chain reaction that leads to the final response.

Receptors are generally found in two cellular compartments: embedded in the plasma membrane (membrane receptors) or in the cell cytoplasm (intracellular or nuclear receptors). These two can be discriminated by the nature of their ligands, since signaling molecules that bind to the intracellular receptors must have a lipophylic profile, allowing them to cross the extracellular membrane and diffuse in the cytoplasm; generally they are lipophilic hormones like steroid hormones and estrogens. The main attribute of nuclear receptors that differentiates them from other classes of receptors is their ability to directly interact with genomic DNA when activated by their ligand. The activation indeed allows them to traslocate in the nucleus and act as typical transcription factors, regulating and controlling the expression of a specific subset of genes.

Classical estrogen receptors belong to this class of receptors. This has been knows since the revolutionary studies of Elwood V. Jensen in the '60s, when he observed that tritium-labeled estradiol injected *in vivo* was selectively retained in the target tissues and withdrawn from the bloodstream (Jensen 1962). This specific uptake suggested that these cells must contain binding proteins, which he called "estrogen receptors." This theory, rejected as first, was eventually validated and accepted in all the scientific community, particularly after Toft's isolation and characterization of the receptor from a crude tissue (Toft & Gorski 1966).

Structure

Following Jensen's studies, researcher soon found that other steroid hormones, such as testosterone, progesterone and cortisone, worked binding to specific intracellular receptors. In the late '80s some studies strengthened this idea and established that the intracellular estrogen receptor was just part of a large family of structurally related nuclear receptors, now known to consist of 48 members (Giguere et al 1988) including steroid receptors (SRs), thyroid/retinoids receptors (TR, RARs and RXRs), vitamin D receptors (VDR), LXR, PPARs, estrogen receptors (ERa and ERb), and orphan receptors for which no ligand has been yet identified. From an evolutionary point of view, it is possible to classify this superfamily members in six different classes (Laudet 1997); to the third family belong the steroid receptors: estrogen receptors (ERα ad ERβ), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR), androgen receptor (AR) and the estrogen-related receptors (NuclearReceptorsNomenclatureCommittee 1999).

Given their common origin, it is not surprising that they share a very similar structural organization. These proteins share indeed the same modular organization, composed of a single polypeptide chain that is divided in at least four distinct functional domains, denoted A to F (Fig. 1), responsible for DNA binding, hormone binding, and transactivation.

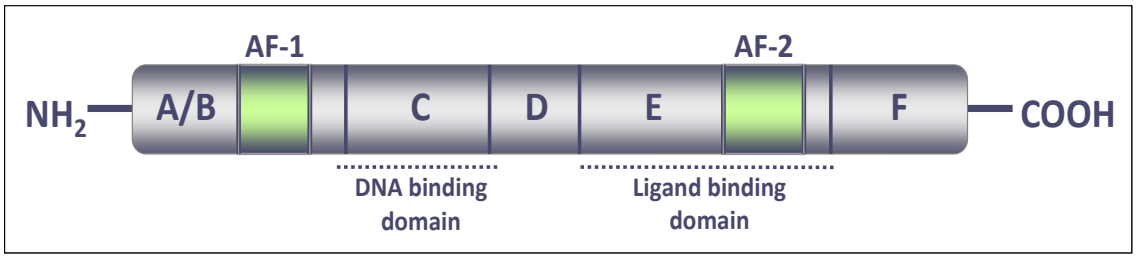


Figure 1 - Structural organization of nuclear Estrogen Receptor

The N-terminal region (A/B domain) is the less conserved domain of the protein during evolution (Krust et al 1986), and represents the most variable region among the nuclear receptor superfamily, with length spanning from 50 to 500 amino acids. It contains an activation function domain (AF-1), which is mainly involved in activating or stimulating transcription in a ligand-independent way by interacting with other components of the transcriptional machinery (Horwitz et al 1996). Indeed many studies have reported that phosphorylation of the receptors in the A/B domain modulate their transcriptional activity in absence of estradiol: the MAPK pathway, the phosphatidylinositol 3-kinase (PI3-K)/AKT pathway and the cyclin-dependent kinases (CDKs) pathway can all trigger the hormone-independent activation of the receptor (Campbell et al 2001, Kato et al 1995, Kato et al 2000, Rogatsky et al 1999). Moreover, the AF-1 domain of ERα recruits several co-activator proteins, some that bind uniquely to this domain (Endoh et al 1999, Wu et al 2001).

The most highly conserved region in the nuclear receptor proteins is the DNA binding domain (DBD), spanning the C region; in fact this 84 amino acid portion appears to be 100% conserved between human and chicken ERα (Krust et al 1986). NMR and X-ray diffraction studies of some nuclear receptors allowed to reveal the 3D structure of the DBD, and to shown that this core domain of 66 base pairs consists of two interdependent zinc finger-like motifs, with eight cysteines that constitute the tetrahedral coordination of two zinc ions, with an amphipathic α-helix at the C-terminal region of each finger (Schwabe et al 1990). On the C-terminal side of the first zinc finger lays the DNA recognition portion, with conserved lysine and arginine residues allowing the selective recognition of the DNA elements. Particularly, studies have indicated that recognition of the estrogen receptor hexameric binding site, characterized by the presence of the AGGTCA motif, is largely determined by three amino acids within the DNA recognition α-helix referred to as the "P-box" (Schwabe et al 1993). The α-helix following the second finger folds over the DNA recognition α-helix to stabilize the structure through hydrophobic interactions and provides phosphate contacts to the DNA backbone.

The DNA binding domain is connected with the ligand binding domain through an extremely flexible peptidic region, called for this reason “hinge”. For its particular localization and properties was believed to function merely as linker, able to bend and to alter its conformation in order to allow the association between C and E domains and to favor proper DNA binding and dimerization (Khorasanizadeh & Rastinejad 2001). However, subsequent studies showed that this region plays a major role in influencing intracellular trafficking and subcellular distribution of the steroid receptors, thanks to the presence of a nuclear localization signal (NLS) sequence.

The hallmark of a nuclear receptor is its ligand-binding domain (LBD, E region), that correspond to the largest domain. Despite a moderate conservation in sequence identity, the three-dimensional structures of the LBDs in the NR superfamily appear to be very similar (Tanenbaum et al 1998). This domain is highly structured and is known to be associated with a wide array of functions, most of which operate in a ligand-dependent manner. The 12 helices identified in the ERα LBD are arranged in an antiparallel α-helical 'sandwich' fold in which three anti parallel alpha helices are flanked by two alpha helices on one side and three on the other. Estradiol is anchored to the ligand pocket thanks to the interaction with an antiparallel β-sheet and the helix H12, known to be directly involved in the transactivation function AF-2 by mutagenesis studies (Danielian et al 1992). Notably, the allosteric changes induced in the receptor showed to be directly influenced by the nature of agonistic or antagonistic ligands that might bind to the LBD; the structural conformation leads to the different ability of the receptor to exert positive or negative effects on the expression of target genes (Moras & Gronemeyer 1998). In fact, partial and pure antagonists induce conformations of the AF-2 region that are distinct from that observed in the presence of pure agonists like estrogen (Shiau et al 1998). The crystal structure of an ERα LBD interacting with its natural ligand showed that the protein conformation modify itself in order to eventually create an highly hydrophibic environment where the lipophylic steroid molecule is shielded and docked (Brzozowski et al 1997).

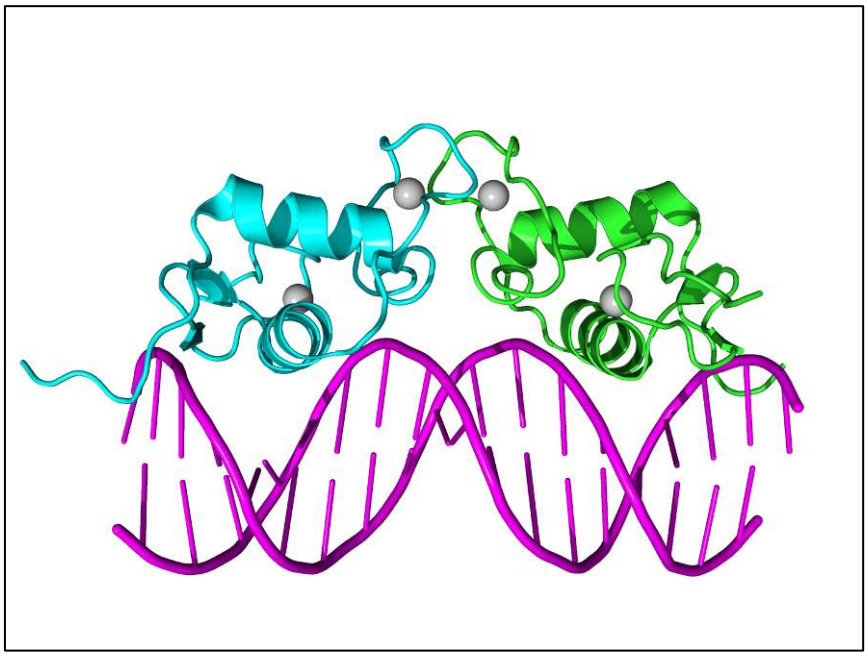


Figure 2 - Nuclear Receptor DNA Binding Domain (DBD) interacting with the double helix

The F domain is designated as the last 45 base pairs at the C-terminus of ERα. This region is extremely variable, and its function and properties are still to be fully elucidated since relatively few studies have been directed to this domain. Notwithstanding, this region seems to play an important role in the activity of the receptor in response to estrogens and antiestrogens: recent studies pointed out that specific point mutations and small deletions in this region were able to alter the response to E2 and tamoxifen (Koide et al 2007, Skafar & Koide 2006).

Mechanism of action

Genomic Actions of Estrogen Receptor

Ligand-dependent activation, direct binding to DNA

Monomers of estrogen receptor in the inactivated form are mantained in the citoplasmatic compartment thanks to its heterodimeric interaction with the heath shock protein complex (HSPs). The most widely-studied HSPs are Hsp60, Hsp70 and Hsp90

(named by their molecular size of 60, 70 and 90 kilodalton, respectively). Particularly, Hsp90 has been extensively studied and proved to play a regulatory role by controlling several of the ligand-inducible functions of receptor. Hsp90 function appears to be related to receptor folding, especially of the hormone binding domains: in vitro studies on estrogen receptor demonstrated that the LBD is sufficient for forming a complex with hsp90, but that complex is much less stable than the similar GR or PR LBD-Hsp90 complexes; in fact, an additional region within the ER nuclear localization sequence region participates in stabilizing the interaction (Picard et al 1990). In the genomic pathway, mediated by the intracellular ERs, hormone binding stimulates a conformational change that triggers Hsp90 dissociation, and receptor dimerization, nuclear traslocation, interaction with co-activators, DNA binding and target gene activation. The canonical model for ER-mediated regulation of gene expression involves the direct binding of dimeric ER to DNA sequences known as estrogen response elements (EREs), which are specific, inverted palindromic sequences. The consensus palindromic element ERE was initially described based on the estrogen-responsive sequence in the *Xenopus laevis* vitellogenin A2 promoter: 5'-GGTCACAGTGACC-3' (Walker et al 1984). Was then demonstrated that human full EREs can be generally described as a 13 bp palindromic inverted repeat with a minimum of 3-base pair spacer: 5'-AGGTCAnnnTGACCT-3' (Gruber et al 2004) As shown by footprint and gel retardation experiments, these particular sequences can be specifically recognized by Estrogen Receptors and ERR (*estrogen related receptors*), so called indeed for their affinity to EREs sequences (Klein-Hitpass et al 1988). This ERE sequence was shown to act on a heterologous promoter in an orientation- and distance-independent manner; at the time of their discovery was not clear if the localization on the genome of those sequences was conserved or not: they appeared to be localized both upstream the transcription start site (TSS), in intergenic region or even overlapping the transcribed region (Sathya et al 1997). However, the majority of the investigation about the localization of the binding sites focused primarily on promoter regions. Only recently, thanks to the novel methodologies available, some studies were

able to expand on these analyses to map estrogen receptor binding sites in a less biased way, which did not depend on preexisting concepts of classic promoter domains. Thanks to chromatin immunoprecipitation combined with microarrays (ChIP-on-chip) techniques, Carroll and colleagues performed a genome-wide analysis of estrogen receptor binding regions in MCF-7 human cells. Their results show that the promoter-proximal regions, although important for some genes, do not constitute the majority of estrogen receptor target sites (Carroll et al 2006). In fact, the majority of estrogen-regulated genes does not have an estrogen receptor binding sites within 50 kb of the TSS, and may therefore act as distant enhancer for the control of target genes transcription.

Ligand-dependent activation, indirect binding to DNA

Another explanation for the fact that the majority of estrogen-regulated genes do not have an estrogen receptor binding sites within 50 kb of the TSS might derive from the fact that nuclear receptors for estrogens can also regulate gene expression without binding directly to DNA. Indeed, ERα is assumed to act through specific protein–protein interactions with other classes of transcriptional factors to affect gene transcription; this imply that, in specific locations in the genome, the receptor can be indirectly bound to non-classical ERE sequences, like Sp1, AP-1 and NfKB. One of the more extensively studied interaction is between ERα and Sp1 (specificity protein 1), which have been demonstrated in several promoters that contain GC-rich elements, such as E2For Bcl-2 (Wang et al 1999). Particularly, GC-rich elements cooperate with ERE-half sites to promote the expression of genes, and mutations of either the GC-rich or the ERE- half resulted in the loss of hormone activation of this construct.

Other studies showed that estrogen-liganded ER is present within the complex of proteins at the AP-1 sites that bind the Jun/Fos transcription factors. The specific role of ERα transcription factor appears to increase the intrinsic transcriptional activity of Jun/Fos when bound to the site (Gaub et al 1990).

Eventually the binding of ERα, either directly bound to DNA or when in complex with other interacting transcription factors, stabilizes the transcription-initiating complex and leads to the RNA Polymerase II and other general transcription factors recruitment. The transcription-initiating complex is assembled on target genes promoter, in a peculiar region called TATA box, a DNA sequence that indicates the point at which a genetic sequence can be read and decoded. In *in vitro* experiments with isolated general transcription factors, TFIID is the first factor to bind to a TATA-box promoter; this multicomponent protein consists of a DNA binding subunit that recognizes the TATA element and is therefore designated TATA-binding protein (or TBP), as well as several TBP-associated factors (or TAFs). Once TBP has bound to the TATA box, TFIIB can bind and recruit the polymerase over the start site. Finally all the other components docks on the complex; in particular, the TFIIH subunits plays an important role in the transcription start, thanks to its helicase activity allowing Pol II to form an open complex and begin the process (Workman & Roeder 1987).

In conclusion, is worth to remember that various ligands other than estradiol share the ability to activate the receptor in different ways. Drugs now known as SERMs (Selective Estrogen Receptor Modulators), such as tamoxifen, bind ER, dissociate heat shock proteins, and induce receptor dimerization and binding to ER responsive sequences nearby target genes (Osborne et al 2000). However, the conformation of the receptor is different when bound by SERMs, and tamoxifen-bound receptor conformation leads it to associate with a different set of coregulatory molecules; the receptor interacting with alternative ligands therefore behaves differently if compared with the estrogen-bound protein. The agonist/antagonist profile of tamoxifen and other SERMs may in part be related to the particular milieu of coactivators and corepressors in a cell. Different response elements in the promoter of target genes may also contribute to the agonist/antagonist properties of these drugs.

Ligand-independent activation

Beyond the canonical model for estrogen-bound ER mediated regulation, several nuclear receptors (e.g. ER, AR, PR, RXR, RAR, and VDR) can be apparently activated in the absence of their cognate hormone by a mechanism referred as ligand-independent activation. Some molecules can indeed trigger signal transduction pathways after their interaction with specific receptors on the outer cellular membrane, and induce the activation of nuclear receptors by mechanisms such as phosphorylation-based processes. This is because ER, as many other nuclear receptors, is a phosphoprotein (Weigel 1996). Several laboratories to date have contributed to the identification of phosphorylation sites in the ER, both serine phosphorylation sites and tyrosine phosphorylation sites: Ser^{104}, Ser^{106}, Ser^{118}, Ser^{167} and Tyr^{537}.

ERa is hyperphosphorylated also in response to the classical ligands including estradiol; some studies suggest Ser^{118} as the major estrogen-induced phosphorylation site (Joel et al 1995, Le Goff et al 1994) even if another study indicates Ser^{167} as the predominant site phosphorylated in response to hormone (Arnold et al 1994, Patrone et al 1998). This discrepancy might be attributable to cell type-specific differences (COS-1 cells *vs.* MCF-7 cells) in ER phosphorylation or reflect differences in the techniques used to map these residues. Tyr^{537}has been suggested to be a basal phosphorylation site, not dependent of the stimulation with hormone (Arnold et al 1995). However, its role in receptor-mediated transcription is unclear; some studies suggested that phosphorylation of this residue influence the ability of the receptor to dimerize and bind DNA (Arnold et al 1995).

The first evidence supporting the existence of this mechanism was provided in the early '90s by Denner and his colleagues, who noticed that l'8-Br-cAMP, a stimulator of cAMP-dependent protein kinase PKA, and okadaic acid, an inhibitor of protein phosphatases 1 and 2A, could lead to Progesterone Receptor activation in absence of the progesterone itself (Denner et al 1990).

Growth factors, Protein kinase A activators (PKA), neurotransmitters and cyclines are all molecules able to activate ER-mediated transcription thanks to the phosphorylation of the receptor.

There is overwhelming in vitro and in vivo evidence that growth factors, triggering the activation of MAP kinases pathway, lead to the ER phosphorylation. First evidences that MAPK could stimulate ER transcriptional activity in a ligand-independent way comes from observations that EGF and IGF-1 can stimulate ER reporter gene expression in cultured cells estrogen-deprived (Bunone et al 1996, Ma et al 1994). In our lab has been demonstrated that other growth factors like TGFα, insulin and insulin-like growth factors (IGF-1 and IGF-2) share the ability to activate ERα. In SK-ER3 cell line, human neuroblastoma derived and stably transfected with the receptor, these factors are able to induce a phenotypic differentiation via a pathway involving MAP kinases (Ma et al 1994).

Activation of ERa by growth factors generally leads to phosphorylation of Ser^{118}, since experiments demonstrated that mutation of this residue to an alanine resulted in loss of EGF-induced activity (Bunone et al 1996). Further investigations definitively proved that growth factors Ser^{118} phosphorylation was mediated by MAPK pathway. Kato et al. indeed showed that activation of MAP kinases enhances ER phosphorylation on Ser^{118} ; moreover, the overexpression of Ras, an intermediate in the MAP kinase pathway, is able to stimulate the activity of wild-type ERs and not Ser^{118} mutants (Kato et al 1995).

Notwithstanding, another study suggests that EGF-mediated activation of ERa might also occur through phosphorylation of Ser^{167} by $pp90^{rsk1}$ within the AF-1 (Joel et al 1998). The importance of Ser^{167} phosphorylation has been shown by the diminished transcriptional ability in response to activation of the MAPK pathway of an ERα mutant, which has Ser^{167} replaced by Ala, compared to the activity of the wild type ERα.

It is of interest to note that the kinetics of Ser^{118} phosphorylation induced by estradiol binding and activation of the MAPK pathway are quite different: in MCF-7 cells, estrogen induces a steady state phosphorylation of Ser^{118} within 20 min, whereas

in response to epidermal growth factor (EGF) or phorbol 12-myristate 13-acetate (PMA), Ser^{118} is rapidly but only transiently phosphorylated (Joel et al 1998).

Coactivators also play an important role in the ability of growth factors to activate ERa. The SRC/p160 family coactivators, AIB1 and GRIP1, can be phosphorylated by MAPK (Erk2) in vitro; in addition, the p300 general coactivator could be co-immunoprecipitated along with AIB1 in a MEK1-dependent manner suggesting this signaling enhanced protein-protein interaction. Moreover, histone acetyltransferase (HAT) activity was detected in anti-AIB1-immunoprecipitated complexes prepared from cells expressing the active MEK1. Taken together, these results suggest that MAPK phosphorylation of these p160 coactivator family members serve to enhance their interaction with p300/CBP and possibly other coactivators, and can therefore lead to indirect regulation of receptor-dependent transcription (Font de Mora & Brown 2000).

In addition, ERα can be activated following the increase of intracellular cAMP and thus via the Protein Kinase A (PKA) signaling pathway. Studies in MCF-7 cells proved that endogenous ERα target genes can be stimulated either with the cAMP analog 8-bromo-cAMP or a combination of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) and cholera toxin, leading to an increases in cAMP production via a G protein-mediated signal transduction pathway. Importantly, in these experiments as well as in later studies the cAMP-induced responses are inhibited by treatment with the pure antiestrogen ICI 164.384, signifying their receptor dependence (9027326). Transient transfection studies indicated that cAMP activation of ERa does not require either Ser^{118} phosphorylation or the receptor's A/B domain. Indeed, an ERa deletion mutant lacking its entire amino terminus is capable of being phosphorylated to the same extent as the full-length receptor (Le Goff et al 1994).

However, the cAMP-mediated signaling pathway has sown to be able to increase the association of ER with cyclin D1, a cell cycle regulator, suggesting that the receptor enhanced activity may be a result of improved interaction with other coactivators (Lamb et al 2000).

Non-Genomic Actions of Estrogen Receptor

In contrast to these relatively well-characterized mechanisms for genomic steroid action, a growing body of evidence suggests that steroids have cellular effects which are not mediated by the transcriptional activation of their cognate nuclear receptors. The classical model of nuclear receptor activation indeed cannot be reconciled with the increasing evidences suggesting that steroid hormones can also initiate signaling from the exterior of the cell, and may in fact use signal transduction pathways commonly employed by other extracellular signaling molecules such as growth factors. These rapid, steroid-mediated effects are referred to as non-genomic signaling responses and can be distinguished from those mediated by transcription. Non-genomic steroidogenic signaling is usually characterized by several criteria: 1) signaling responses occur within a time course (seconds to minutes) that is generally considered too rapid for transcriptional and translational events to take place; 2) responses are refractory to inhibitors of transcription (e.g. actinomycin D) and translation (e.g. cycloheximide) and 3) responses can be initiated by steroids conjugated to macromolecules (e.g. bovine serum albumin, BSA) which are theoretically too large to enter cells.

Nongenomic effects have been reported for many classes of steroids (e.g. progestins, estrogens, androgens, mineralocorticoids, and glucocorticoids), as well as the secosteroids and thyroid hormones. Each of these classes of ligands exhibits broad and varied nongenomic responses which include modulations in the activity of signal transduction cascades, ion transport and neurotransmitter release.

Membrane estrogen receptors

Growing evidences suggest that the rapid action of E2 involves a nuclear ER acting at the level of the plasma membrane. Using antibodies directed against different epitopes

of ERα, several labs have demonstrated its localization in the region of the outer cellular layer (Pappas et al 1995). However, the identity of membrane ER has been to date quite controversial. Just recently, to determine the nature of endogenous E2-binding proteins, an unbiased isolation of membrane ER from a breast cancer cell line was accomplished by affinity chromatography. The estrogen-binding protein was identified by mass spectrometry as the classical ERα, identical to the nuclear ERα from the same cells (Pedram et al 2006). These findings agree with results from many additional cell-based approaches, including siRNA knockdown of classical ERα and ERβ and earlier studies using anti-sense oligonucleotides and immunohistochemical identification of ER at the membrane. Could be estimated that approximately 5-10% of total cellular ER is found at the plasma membrane in many cells, with proportion depending on the cell type. Some laboratories focused on the identification of the ERa structural determinants required for the membrane localization. Serine 522 was first identified to be necessary for ER translocation, as this residue promotes interaction with the caveolin-1 protein (Razandi et al 2003), a transporter of ERα to the caveolae rafts to plasma membrane. Indeed, in cells lacking caveolin-1, endogenous ERα is only found in the nucleus. Moreover, a cysteine in the ligand-binding domain was found to be a site for palmitoylation that promotes association of ER with caveolin-1 (Acconcia et al 2005). Caveolae-localized ERα and ERβ associate with and activate Gα and Gβγ proteins as perhaps the earliest rapid signals generated; this interaction leads to calcium and cAMP generation, and the activation of both proximal kinases (Src, PI3K) and distal kinases (ERK, AKT) (Kumar et al 2007).

Very recently two laboratories have shown that a G-protein-coupled receptor (GPCR30) can act as a distinct membrane ER in human SKBR3 breast tumor cell line (Thomas et al 2005). However, the importance of this receptor is still debated. This receptor was initially reported to respond to E2 or the ER antagonist, ICI 182780, with both binding and signal transduction (e.g. cAMP generation and calcium stimulation). More recent work has addressed this concept definitively: four different GPR30 knockout mice have been created and show little phenotype despite

extensive evaluation (Levin 2009). In some cells types, there is evidence that GPR30 might collaborate with membrane-localized ERα as part of a large complex of proteins at the membrane, transmitting membrane ERα-generated signals to downstream kinase cascades (Vivacqua et al 2006).

However, the majority of non-genomic actions of ER are exerted thanks to their interaction, also called "cross-talk", with other signaling pathways.

Crosstalk with other signaling pathways

Numerous studies have demonstrated that estradiol (E2) can interact with the MAP kinases pathway; indeed it rapidly activates extracellular-regulated kinases (Erk1/Erk2) and can influence c-Jun N-terminal kinase (Jnk) activity (Improta-Brears et al 1999); (Migliaccio et al 1996). This process seems to be directly connected to the ability of estradiol to stimulate the tyrosine kinase activity of Src, a protein kinase upstream the MAPK pathway that plays the role of mediator. Src activated has been demonstrated to interact with ER (ERa or ERb) both *in vitro* and in cells (Migliaccio et al 1996). This interaction takes place between the Src homology-2 (SH2) domain of Src kinase and the LBD of ERa. Moreover, mutation of Tyr^{537} to phenylalanine, located within the ERa LBD, abolishes the receptor's interaction with Src kinase and thus E2-dependent Src kinase activity. The interaction between Src kinase and ERa leads to activation of two Src substrates, Shc and Ras GAP (GTPase activating protein)-associated p190, which in turn leads to activation of ras and theoretically activation of MEK kinases.

Moreover, cell treatment with estradiol can influence another member of the MAPK family, Jnk. This factor has been implicated in various cellular mechanisms leading to an increased apoptosis, in part by phosphorylating and thus inactivating some of the better known antiapoptotic regulators Bcl-2 and Bcl-xl. In contrast to the hyperstimulatory effect that they exert on Erks, E2 and E2-BSA have been described as inhibitors of Jnk activity, causing a diminished apoptosis in human stimulated breast cancer cells (Razandi et al 2000).

Other findings pointed out that treatment with estradiol or with the membrane-impermeable estrogen-BSA can also rapidly stimulate the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA)-dependent signaling pathway in a wide variety of cells. Beyer and colleagues (Beyer & Karolczak 2000) noticed that this treatment was sufficient to promote neurite growth and arborization of dopaminergic neurons in culture. Notably, these effects could be blocked by an inhibitor (H89) of PKA, suggesting that estrogen can interact with membrane binding sites on dopaminergic neurons, thereby stimulating the cAMP/PKA/phosphorylated cAMP-responsive element binding protein (CREB) signalling cascade.

Although the effects of estrogens on PKC activity have not been explored extensively, there is evidence to suggest that E2 can stimulate activity of this kinase signaling pathway. Indeed, the effect of E2 on PKC activity has been observed in the preoptic area of female rat brain slices, but not in the hypothalamus or cortex (Ansonoff & Etgen 1998). Usually, the increase in PKC activity could not be inhibited by the antiestrogen, ICI 182,780, but has proven to be highly attenuated to a phosphatidylinositol-specific phospholipase C (PLC) inhibitor (U73122) (Sylvia et al 2001). The same inhibitor was able to attentuate E2-induced changes in Ca2+, demonstrating the involvement of G-protein-coupled receptor stimulation of phospholipase C in this process (Picotto et al 1999). Notwithstanding, in other studies the PLC inhibitor had no effect, which might reflect cell type-specific differences in these mechanisms. However, the dynamics of Ca2+ signaling must also be considered when comparing these responses. Indeed, while it has been demonstrated that PLC inhibitors can block rapid (< 1 min.) increases in Ca2+ release, a second, presumably PLC-independent, wave (>5 min.) of Ca2+ release was also observed (Picotto et al 1999). However, PKA and PKC are also thought to play roles in these E2-dependent Ca2+ responses. Thus, multiple signaling pathways can influence E2-dependent changes in cytosolic calcium and this likely depends on cellular context as well as other factors.

Coactivators and Corepressors

The observation that the transcriptional activities of ER ligands are evident in a tissue-selective manner suggests that the receptor is not self-sufficient, but rather, requires specific cellular factors for maximal responses. From this observation some group of research postulated the theory of the tripartite action of the ER, involving the receptor, its ligands and coregulator proteins and allowing for the precise regulation of the biological effects of these hormone receptors on gene expression (Katzenellenbogen et al 1996). Furthermore, studies reporting estradiol-ER induction of genes containing no apparent ERE-like sequence led to the discovery that ligand-activated ER can interact in an indirect manner with the regulatory regions of target genes. In fact, ER was shown to inhibit progesterone receptor (PR) and glucocorticoid receptor (GR) activation on promoters lacking EREs (Meyer et al 1989).

Estrogen Receptor Coactivators

The first ER-interacting proteins were identified by Halachmi et al., who used a gluthathione S-transferase (GST) fusion protein incorporating the ERα ligand binding domain LBD to isolate two proteins of approximately 140 and 160 kilodaltons, termed ER-associated proteins 140 and 160, ERAP140, ERAP160 (Halachmi et al 1994). Subsequent studies identified additional proteins that bound the LBD of ER and other NRs, including transcriptional intermediary factor 1 (TIF-1), receptor interacting proteins 140 and 160 (RIP140, RIP160), human receptor potentiating factor 1 (hRPF1), thyroid hormone receptor interacting protein 1 (TRIP1/SUG1), and thyroid hormone receptor associated proteins (TRAPs/DRIPs) (McKenna et al 1999). It was not until the cloning and characterization of the coactivator SRC-1 (steroid receptor coactivator-1) was accomplished, however, that a specific role for this class of proteins in ER action was defined. The SRC-1 protein was initially identified in a yeast two-hybrid system, using the PR LBD as bait, and was subsequently shown to interact with ERα and potentiate its activity in an agonist-dependent manner (Onate et al 1995).

The cloning of SRC-1 led to the discovery of a whole family of structurally and functionally similar coactivators (termed the p160s). These include the murine glucocorticoid receptor-interacting protein-1 (GRIP1), transcriptional intermediary factor 2 (TIF-2; human homolog of GRIP1), and amplified in breast and ovarian cancer-1 (AIB-1). The SRC/p160 family members share a common structure, most noticeable in the N-terminal domains, which contain conserved helix-loop-helix and PAS (per/ARNT/sim) domains thought to mediate homo- and heterodimerization (McKenna et al 1999). The conserved LXXLL (LXXLL; L = leucine, X = any amino acid) motif facilitates the interaction of SRC coactivators to liganded nuclear receptors and three copies of this sequence are located centrally within coactivator proteins; these structural motifs form amphipathic α-helices in which the leucines create a hydrophobic surface that fits into the hydrophobic groove of the receptor AF-2 domain. Many studies revealed that the sequences flanking the LXXLL motifs are key determinants of the binding affinity and specificity of coactivators for their NR partners (McInerney et al 1998). Finally, a transactivation domain and an intrinsic histone acetyltransferase activity domain maps to the coactivator C-terminus, giving them an intrinsic histone acetylase activity (HAT), known to facilitate chromatin remodeling at target promoters (Spencer et al 1997).

After hormonal activation, the estrogen receptor, through its LBD, interacts directly with both a p160 coactivator family member and the cointegrator, p300/CBP, and this is followed by recruitment of P/CAF (p300 and CBP-associated factor) (Ogryzko et al 1996). Both p300/CBP and P/CAF display histone acetylase activity and contribute to the remodeling of selected chromatin domains within the promoters to which they are targeted.

In addition to the coactivators that enhance receptor activity by interacting with the LBD, AF-1-interacting coactivators have also been described. This might be inferred, for instance, from the fact that tamoxifen inhibits the AF-2, but not the AF-1 activity of ERα, and displays estrogenic agonist and antagonist activities in a tissue-specific manner; particularly, the partial agonist activity of tamoxifen is most prominent in cells

where AF-1 is the dominant activation function (Berry et al 1990). Specifically, steroid receptor RNA activator (SRA) is an RNA transcript that enhances the AF-1 activity of ER and other steroid receptors; it is observed in large riboprotein complexes that contain SRC-1 (Lanz et al 1999), probably facilitating SRC-1 recruitment to steroid receptor. Moreover, Endoh et al. have characterized a p68 RNA helicase that potentiates the activity of ERα AF-1 in both the estrogen- and antiestrogen (i.e., estrogen antagonist)-liganded receptor (Endoh et al 1999). The p68 protein may play a role as adaptor in the recruitment of AF2 coactivators, as suggested by in vitro studies that detected the association of p68 with p300/CBP.

Table 1_List of ERa coactivators (Table from (Hall & McDonnell 2005)

	CoFactor	Full Name	Other Names	Function	Interaction with ER
AF-2 Coactivators	SRC-1 (p160)	Steroid receptor coactivator-1	NCoA-1	HAT (Histone acetyltran-serase)	Binds ERs AF-2 through LXXLL motifs
	SRC-2 (p160)	Steroid receptor coactivator-2	GRIP1, TIF-2, NCoA-2	HAT	Binds ERs AF-2 through LXXLL motifs
	SRC-3 (p 160)	Steroid receptor coactivator-3	AIB1, ACTR, p/CIP, RAC3, TRAM-1, NCoA-3	HAT	Binds ERs AF-2 through LXXLL motifs
	CBP/p300	CREB-binding protein		HAT	Binds ERs AF-2 through LXXLL motifs
	TRAP220, TRAP/DRIP	Thyroid hormone receptor activating protein of 220 kDa	mediator; PBP		Binds ERs AF-2 through LXXLL motifs
	ASC-1 ASC-2	Activating signal cointegrator-1 Activating signal cointegrator-2	RAP250, TRBP, AIB3	Bind HATs and NRs	Binds ERs AF-2 through LXXLL motifs
AF-1 Coactivators	SRA	Steroid receptor activator		Splicing	Binds ERα AF-1
	p68	p68 RNA helicase		RNA helicase	Binds ERα AF-1
Secondary Coactivators	CARM1	Protein methyltransferase 1		Arginine histone methyltransferase	Binds ERs AF-2 indirectly through association with p160s
	PRMT1	Protein methyltransferase 1		Arginine histone methyltransferase	Binds ERs AF-2 indirectly through association with p160s
	CoCoA	Coiled-coil coactivator			Binds ERs AF-2 indirectly through association with p160s
Dual-Functional Coactivators	E6-AP	E6-associated protein		Ubiquitin ligase	Binds ERs AF-2
	RPF-1	Receptor potentiating factor-1		Ubiquitin ligase	Binds ERs AF-2
	PGC-1α PGC-1β	PPARγ coactivator-1		Tethering surface for other cofactors; splicing	Binds the hinge region of the ERs
	CAPER-alpha CAPER-beta CoAA	Coactivator of (AP-1) and estrogen receptors Coactivator activator		Potentiate ER activity; splicing	Associate with ER indirectly through binding ASC-2

In addition to coactivators that bind the ERs in a direct manner, a series of factors have been described that enhance ER activity through their ability to interact with the p160 coactivators, thus affecting ERs indirectly. For instance, the p160s, besides LXXLL motifs that allow for ER binding, contain also other domains located at the C-terminal (AD1 and AD2) and N-terminal basic-helix-loop-helix/PAS (bHLH/PAS) domains, which can associate with other factors involved in chromatin remodeling. Specifically, AD1 recruits the histone acetyltransferases CBP and p300, and AD2 interacts with protein arginine methyltranferases (PRMTs) such as coactivator-associated arginine methyltransferase 1 (CARM1) and PRMT1 (Chen et al 2000).

Estrogen Receptor Corepressors

To provide the necessary regulation of Estrogen Receptor activity in all the tissues where it exerts its functions, corepressor proteins have been identified that reduce the agonist effects of estrogens. Best characterized among these are N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptors), structurally related corepressor proteins present as components of larger complexes that also contain histone deacetylases (Chen & Evans 1995, Horlein et al 1995). Both N-CoR and SMRT bind tamoxifen-bound ERα via interaction sites within the hinge domain (D region) (Chen & Evans 1995) and suppress receptor activity through repressor domains located within their N-terminal regions (Jackson et al 1997). The interaction of these corepressors with ER LBD is mediated by two NR-interacting domains (CoRNR boxes) found within both NCoR and SMRT, which contain sequences that are similar to those found in the NR boxes (LXXLL motifs) of coactivators (Hu & Lazar 1999).

Like coactivators, NCoR and SMRT interact with secondary cofactors that enable them to manifest their repressive effects on NRs. The repressor domains of NCoR and SMRT bind mSin3, a protein that associates with HDACs (Hu & Lazar 2000). Tissue-specific repression of NRs is also mediated by a secondary cofactor, mSiah2, which targets

NCoR for proteasomal degradation. The amount of mSiah2 found in cells varies according to the cell type, and its expression is inversely correlated with cellular NCoR content and degree of NR transcriptional repression (Zhang et al 1998).

Regulation of ERα Cofactors

As described above, ERα can be activated by extracellular and ligand-independent signals and enhance target gene expression even in the absence of the natural hormone. However, not all of the ligand-independent activities of ERs affected by extracellular pathways can be attributed to direct kinase phosphorylation of the receptors; in fact, this regulative mechanism seems to affect also the activity of coregulator proteins. Given that most cofactors exist in similar amounts in all tissues, it is reasonable to anticipate that there may exist mechanisms to turn on and off their activities to provide more sensitive control of transcription. Accordingly, coactivators were recently found to serve as points of convergence between ER and growth factor pathways: SRC-1, GRIP1, and AIB1 are phosphorylated by MAPK, an event that enhances their activities (Smith & O'Malley 2004). However, other studies indicated the presence of some cofactors whose activity was not enhanced by phosphorylative events: for instance, MEK-1-mediated phosphorylation of SMRT effectively causes a loss of repression by promoting disassociation of SMRT-TR complexes (Hong & Privalsky 2000); thus, extracellular signaling pathways may enhance NR action by coordinately enhancing the recruitment and activity of coactivators while decreasing the association and functionality of corepressors.

Estrogen Receptor Binding Sites

Estrogen receptor dimers bind to the canonical 13-bp ERE, GGTCAnnnTGACC, a palindromic inverted repeat (IR) separated by any three nucleotides (nnn) identified in 1986 in the promoter of the Xenopus vitellogenin gene, thanks to conserved sequence

alignments (Klein-Hitpass et al 1988, Walker et al 1984), and subsequent transfection experiments showed that this element also functions in human cells. The data collected in the next experiments confirmed the 13 bp palindrome, but the consensus ERE sequence was subsequently extended to a 15-bp palindrome (AGGTCAnnnTGACCT) when the flanking sequences were noted to contribute to dimer-binding affinity (Klinge 2001).

Once full human genomic sequence data became available, several groups of investigators began a computational search of EREs sequences in the genome, to localize all the possible binding of the receptor on the DNA double strand. This was obtained with novel bioinformatic approaches based upon the concept of position weight matrices (PWM), which can be derived from a set of known binding site sequences, by simply tabulating the nucleotide count at each position and visualizing the differences of the nucleotide conservation. The weight matrix table shows the frequencies of the nucleotides (A, C, G, T) at each position of the aligned sequences.

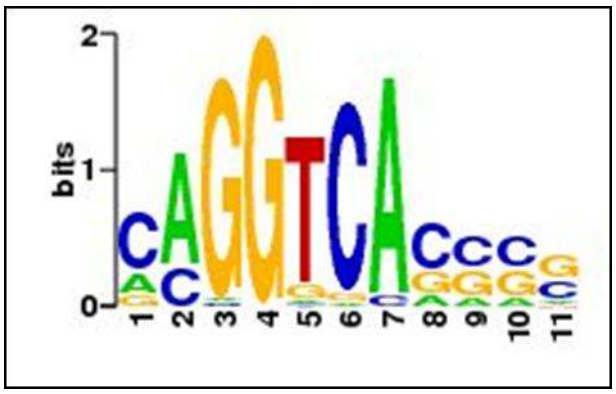


Figure 3 - Position weight matrix of ERE-half site

This approach led to the observation that about 70.000 EREs are present in the human genome, one in every 43 kb of DNA (Bourdeau et al 2004). Moreover, allowing up to 2-bp substitutions from the consensus ERE, revealed >17.000 and >15.000 possible EREs within 15 kb of annotated transcription start sites, respectively. Yet, chromatin immunoprecipitation studies in MCF7 cells have revealed that only 1000–10.000 loci are bound by ERα in response to estrogen treatment (Carroll et al 2006), and therefore a

consistent number of alleged ERE candidates remains unbound in the experimental conditions considered. Moreover, different studies indicated that about 50% of all ERα-bound sequences do not have a recognizable ERE and therefore might correspond to sites of ERα tethering via other transcription factors or contain atypical estrogen response elements, like tandem half-ERE sites (Bourdeau et al 2004). In fact it has been shown that different motifs, including transcription factor Sp1 and AP1 (activator protein 1), are important regulatory motifs for ER-mediated transcription. Recently the Forkhead-binding motif has been identified as being of potential importance in ER-mediated transcription based on ChIP-on-chip analysis (Carroll et al 2006, Laganiere et al 2005), and Sp1 sites and GC-rich sites have been shown to mediate transactivation of a large number of estrogen-responsive genes in breast cancer (Safe 2001).

Not only the identity of ER binding sites have been investigated in recent years, but the analysis of the localization of the estrogen receptor-bound sequences has gained the same interest. Location analysis for ERα-binding sites throughout the human genome revealed a substantial long-range (>10 kb) enhancer functions of the receptor. More than half of the binding sites indeed is not positioned immediately nearby annotated genes and their transcriptional start sites (Carroll et al 2006). For this reason, many techniques have been developed to investigate long-range chromatin interactions between DNA elements engaged in transcriptional regulation, like chromosome conformation capture (3C) and its variants, FISH (fluorescence *in situ* hybridization) and ChIA-PET (Fullwood et al 2009). With this methodology was observed that ERα protein dimers are recruited to multiple and distal ERα binding sites, which are allowed to interact with one another and possibly with other factors such as FoxA1 and RNAPII to form chromatin looping structures around target genes.

3. Physiological role of Estrogen Receptors

Role in the reproductive system

The structure and functionality of the whole reproductive system are deeply influenced by the estrogens action. Their involvement is of primary importance during all the phases of the life cycle, especially of female individuals: during organogenesis, when an excess or a shortage may cause alterations and ovary malfunctions (Abbott et al 2006); during puberty, when the whole reproductive system is cyclically modified by estrogenic and progestinic blood levels; during the women fertile period, when sexual hormones are responsible for the control over the reproductive function; in the menopause when the block of ovary follicles production totally prejudice the reproductive behavior.

Ovary

The ovary in one of the main reproductive organs influenced by the estrogenic activity, and is a key organ for the maintenance of the reproductive system and of the mammary gland functions. It is responsible indeed for two key activities: the gametogenic activity, allowing the generation of female aploid gametes, and the endocrine activity, being the main source of estrogen in women.

From a morphological point of view, the ovary can be divided into three functional units: follicles, corpus luteum and stroma; each of them is able to synthesize sexual hormones in response to gonadotropin stimuli. Follicles, again, are constituted by three types of cells: theca cells, granulosa cells and germinal cells (oocytes). Corpora lutea are made by differentiated theca and granulosa cells at their terminal stage.

Theca and granulosa cells are extremely different, under many aspects. As regards the hormone production, the first ones are dedicated to the androgen production, while

the others convert androgens of thecal origins in estradiol, thanks to an aromatization process. Indeed the two kinds of cells express different sets of steroidogenic enzymes and this differential pattern of enzymes is regulated by pituitary hormones. Moreover, the localization of the different ER isoforms is distinct in the two types of ovary cells, with ERα predominantly expressed in theca and stromal cells, while ERβ, the most abundant ovarian estrogen receptor, is mainly found in granulosa cells, but it is also expressed in luteal cells and, to a lesser extent, in thecal cells (Fitzpatrick et al 1999, Guo et al 2001, Pelletier et al 2000).

Classically, two approaches have been used to study the role of estrogen within the ovary: surgical removal of the pituitary gland to eliminate confounding gonadotrophins, and pharmacological blockage of the synthesis of estrogen both *in vivo* and *in vitro*. In fact, much of our understanding of the physiological roles of oestrogens has come from studies in which circulating oestrogen is depleted by ovariectomy and replaced with exogenous treatments. However, such approaches do not discriminate between responses modulated by ERα, ERβ, or via another mechanism; for this reason, in recent years, transgenic gene knockout technology has been used to investigate the intraovarian effects of estrogen, and were generated mice lacking one of both the receptor (αERKO and βERKO), as well as mice that lack the ability to synthesize oestrogens due to deletion of the aromatase gene (ArKO).

Ovaries of αERKO mice does not present any important different at birth compared to controls, supporting the hypothesis that estrogen is not involved in the germ line maturation in the foetal life (Schomberg et al 1999). However, with the begin of the sexual activity, αERKOs females show a distinct phenotype characterized by acyclicity, infertility and hyperemic ovaries devoid of corpora lutea. Folliculogenesis is arrested at the antral stage with large follicles becoming cystic and haemorrhagic (Lubahn et al 1993). Since synthesis and secretion of pituitary gonadotropins is strictly regulated by steroids of ovarian origin, lack of ERα cause disruptive effects at hypothalamic-hypophisary level: the main effect is a chronic and sustained LH secretion. Indeed, prolonged administration of a gonadotropin releasing hormone (GnRH) antagonist to

αERKO mice prevented formation of the haemorrhagic cysts indicating that the ovarian phenotype manifests as a consequence of elevated LH levels (Couse et al 1999, Hohmann et al 2005).

The female βERKOs have small ovaries, some arrested follicular development and their fertility is compromised with reduced numbers of offspring/litter, consistent with the reduced numbers of corpora lutea observed (Emmen & Korach 2003, Krege et al 1998). Gonadotrophin levels are normal in these mice.

The generation of double ER knockout (αβERKO) mice by two laboratories (Couse et al 1999, Dupont et al 2000) and reports of their ovarian phenotype, indicate that these mice are distinct from the individual ER knockouts. These ovaries exhibit follicular trans/re-differentiation with tubular-like structures containing Sertoli-like cells. Where oocytes were present these were seen to be degenerating, raising the possibility that factors produced by the oocyte may be involved in the transformation process. The phenotype is expressed in the presence of elevated LH levels, similar to that of the ERKO mouse.

Thus, it appears that both ERs have roles to play in the maintenance of fertility and ovarian function.

Uterus

The uterus, a major target tissue for ovarian hormones, is composed of heterogeneous cell types (stroma, luminal epithelial, glandular epithelial, and smooth muscle), that undergo continuous synchronized changes of proliferation and differentiation in response to changes in levels of circulating estrogen and progesterone (Li 1994, Martin et al 1973). Estrogen, by regulating estrogen target genes in a cell-specific manner, has different effects on different types of cells in the uterus. In the immature (21-day-old) mouse uterus, the cellular proliferation rate is very low. Proliferation is initiated at puberty in response to cycling estrogen, although the immature uterus is fully capable of responding to E_2, and estrogen induces both epithelial and stromal cell proliferation.

Although ERα is highly expressed in the epithelium, is still debated if proliferation of epithelial cells in response to E_2 is a direct or indirect mechanism, probably mediated by growth factors secreted by stroma in response to estrogen are the mitogens in the epithelium.

Preliminary experiments seem to foster the hypothesis of non-mediated estrogenic action, since has been observed that estradiol possess the ability to directly stimulate endometrial cells proliferation in the uterine horn, with no effects on the controlateral horn (Stack & Gorski 1984). However, the same hormone treatment has no effect on the proliferation of cultured endometrial cells, suggesting the involvement of other trophic factors.

This apparent contradiction has been resolved with the observation that estrogen receptors are not expressed in the endometrial epithelial cells, albeit they can be observed in the adjacent stroma: endometrium proliferation, therefore, may be induced by a paracrine mechanism by growth factors secreted by stromal cells in response to estrogen action (Pierro et al 2001).

Insulin-like growth factor 1 (IGF-1) is one of the best studied candidates as mediator of estrogenic actions in the uterus, and different sets of experiments support this hypothesis. First, the growth factor receptor are expressed in the whole uterus, especially in epithelial cells, but also in stroma; moreover, experiments showed that IGF-1 transcript is increased in both stromal and epithelial compartments of the uterus by E2, with greater signal apparent in the stroma (Zhu & Pollard 2007). Transgenic mice overexpressing Igfbp1, an IGF-1 binding protein which sequesters and therefore decreases the amount of available IGF1, have an attenuated uterine response to E2 (Rajkumar et al 1996). Additionally, IGF-1 null mice lack a full uterine proliferative response and, more specifically, lack G2/M progression of the epithelial cells following E2 stimulation (Adesanya et al 1999); however, uterine response is restored by transplanting Igf1KO uterine tissue into a WT host (Sato et al 2002). Conversely, *in vitro* studies have demonstrated that estradiol-independent ER transcriptional activity can be induced by IGF-1 signaling, providing evidence for a cross-talk mechanism

between IGF-1 and ER, and suggesting a second level of regulation (Aronica & Katzenellenbogen 1993); the results were observed also *in vivo*, suggesting the deep involvement in IGF-1/ER cross-talk in uterine physiology and establishing that ERα is required for a proper response to E2 or IGF-1 stimuli (Klotz et al 2002).

Mammary gland

In mammals, the mammary gland is essentially undeveloped at birth and does not undergo full growth until the completion of puberty and, in fact, remains undifferentiated until pregnancy and lactation. Development of the mammary gland may be divided into five distinct stages: embryonic and fetal, prepubertal, pubertal, sexually mature adult, and pregnancy/lactation (Imagawa W 1994).

The involvement of estrogens in the control and in the regulation of mammary gland physiology is extremely complex, and implies interconnections with many different ancillary hormonal factors, necessary for the appropriate development and activity of this tissue. Although the developmental factors involved during the embryonic and fetal stages of the female mammary gland are poorly understood, estrogen action does not appear to be essential; however, studies have shown that the fetal and neonatal mammary gland of rodents is responsive to the gonadal steroids. It has been suggested that estrogen may intervene stimulating epithelium proliferation in its first developmental stages, and that this action may be opposed by testosterone (Mori et al 1979).

The next stages of mammary gland development begin after birth and eventually culminate with a complete mature gland able to lactate. All these last steps are regulated by ovarian sex steroids, which cause the massive and constant development of the mammary ducts.

Ductal elongation is seen indeed in the first few days after birth and originates from a few small terminal end buds (TEBs) and is probably the result of residual effects of maternal and fetal hormones. Although ductal growth is slow for the first 2–3 weeks, it

accelerates greatly with puberty. Estradiol has been shown to act directly on the mammary gland to stimulate ductal morphogenesis during puberty (Daniel et al 1987, Silberstein et al 1994).

Further side-branching of epithelial ducts and formation of alveolar buds occurs in the mature animal under the additional influence of prolactin and progesterone. Estrogens and progesterone are known to promote proliferation and differentiation in the normal breast epithelium. Both steroids act intracellularly through a receptor which, when activated by its binding with the hormone, regulates the expression of specific genes. However, the mechanism by which these molecules exert their mitogenic and differentiation effect has not been clearly established. One of the accepted mechanisms of action of steroid hormones postulates that the proliferation of cells is the response to direct stimulation, as the result of the interaction of the estradiol bound to the estrogen receptor (ER) with the DNA.

Female patients lacking aromatase enzyme, therefore not able to synthesize 17β-estradiol from testosterone, do not show any sign of differentiation in mammary gland during pubertal age. In tese cases, estradiol injection causes a normal mammary development. Studies carried out in ovariectomized female mice highlighted an important role of estradiol, GH (growth hormone) and IGF-1 in the glandular ducts development (Morishima et al 1995). Other hormones take part in the regulation of the process leading to the complete mammary development, such as prolactin, insulin, tyroid hormones and glucocorticoids. Moreover, they mediate the lobular-alveolar development observed during pregnancy; indeed in this period and later during lactation, the gland differentiates in order to create peculiar structures able to secrete milk, the alveoli.

In mammary gland cells the isoform β of the receptor is expressed from the embryonic state; notwithstanding, the phenotypic observation of female knockout mice for this receptor failed to show a significant role for the ductal development. Recently, an ultrastructural analysis evidenced an ERβ role in the epithelial cells arganization and adhesion. This phenomenon appeared to be particularly evident during the lactation

phase, where the lack of the receptor cause a reduction of the secerning epithelium and a dilatation of the intraepithelial distance (Haslam & Nummy 1992, Pendaries et al 2002).

On the other hand, α receptor is expressed both in the epithelial and in the ductal cells, but not in the lobule cells. This receptor is necessary for the estrogenic stimulation of the cellular proliferation and the epithelium growth (Haslam & Nummy 1992, Pendaries et al 2002).

Central Nervous System (hypothalamic-pituitary axis)

Neuroendocrine system evolved with a specific and important function: to integrate the messages received from the external environment with the nervous signals, reflecting the inner body condition, to eventually intervene with appropriate physiological actions.

Most of neuroendocrine systems control homeostasis thanks to a thick web of nervous fibers that controls signals with classical negative feedback mechanisms. Hypothalamic-pituitary axis, though, is characterized also by a positive feedback control exerted by estrogens, responsible for the gonadotropins release peak in the bloodstream. Therefore, ovary estrogen can act with a double effect, dependent on the hormone blood concentration and on the estrous cycle phase.

Ovarian cycle is tightly regulated by pituitary gonadotropins, FSH (Follicule-stimulating Hormone) and LH (Luteinizing Hormone), whose production depends upon the release of gonadotropin releasing hormone (GnRH) (Fink 1979). FSH stimulates ovarian follicles growth and maturation; in these follicles, granulose cells start producing estradiol at low concentration. Later, LH stimulus cause follicles breakage, and the cells face a structural and biochemical transformation that culminates with the corpus luteum generation, able at this point to release massive amounts of progesterone and estradiol.

Low hormone concentration in the first part of folliculogenesis have a suppressive effect on LH release, while the sharp increase of the estrogenic levels during the

proestrus phase stimulate the hypothalamic-pituitary axis inducing the gonadotropins wave triggering ovulation. In ERα knockout animals, LH plasmatic levels are elevated, despite high circulating estrogens, giving a first demonstration of the critical role of the α receptor in the negative feedback regulation on the luteinizing hormone synthesis and secretion; similarly, in female mice lacking aromatase gene the same phenotype can be observed, and the LH concentration can be reduced only after exogenous estradiol injection (Britt et al 2004, Couse et al 2003). Females lacking the isoform β of the receptor do not show any significant variation in LH plasmatic levels, indicating that this isoform involvement in the estrogen negative feedback mediation on the neuroendocrine system is minimal (Couse et al 2003).

Conversely, FSH synthesis and secretion are not affected in ERα knockout mice; however, following ovariectomy they appear to be highly induced, suggesting an involvement of the ovarian hormone in the FSH control. Surprisingly, estrogenic treatments reverts only partially the phenotype observed, supporting the idea that FSH synthesis and secretion may be dependent from inibins/activins peptidic hormones (Gregory & Kaiser 2004).

Role in the non-reproductive organs

Beyond its established role in the regulation of the reproductive activity, a growing body of recent experiments is emphasizing the key role of estrogens and their receptors in non-classical organs.

Bone

Fuller Albright, 70 years ago, made the observations that patients with osteoporosis were almost totally postmenopausal women, with few men and premenopausal women exception (Fuller 1940), supporting the evidences on the protective effects of

estrogen on bone. In fact, estrogens play a major role in the regulation of skeletal growth during development and in the maintenance of bone integrity during adult life. the high circulating levels of estrogen likely have important effects not only on bone but also on extraskeletal calcium homeostasis, such as intestinal and renal calcium handling (Riggs et al 2002).

Growth and bone remodeling result from the balance between two cellular population: osteoblasts, responsible for new material deposition, and osteoclasts, responsible for bone resorption (Cauley et al 1995). Bone strength and resistance is therefore determined by the relative activity of the two cellular types: during development and growth the blastic function prevails, while the clastic function is predominant during aging. Excessive clastic activity may cause osteoporosis, a condition that quickly and abruptly emerge in postmenopausal women; in case of hormone replacement therapy, the problems connected with osteoporosis appear clearly reduced and delayed, indicating a protective estrogenic activity [Lindsay, 1976; Spelsberg, 1999; Christiansen, 1990]. The evidence that estrogen, acting via estrogen receptor ERα, stimulates osteoclast apoptosis (Martin-Millan et al, Nakamura et al 2007) and, conversely, suppresses osteoblast and osteocyte apoptosis (Chen et al 2005, Tomkinson et al 1997) support these data.

Other studies reinforce the hypothesis that estrogens act on bone indirectly, suggesting a close cooperation between the hormone and other factor in the remodeling process. Particular attention requires the combined action of estrogens and growth hormone (GH); indeed some observation indicates that hormone replacement therapy in postmenopausal women increases GH blood levels and GH receptor in osteoblasts, suggesting an interdependency of estrogen and GH functions in the maintenance of bone homeostasis (Bryant & Dere 1998, Bryant et al 1999, Chen et al 1995, Sandstedt et al 1996, Scillitani et al 1997).

Estrogen deficiency is also associated with increases in bone marrow levels of a number of pro-resorptive cytokines, including TNF-α, IL-1α, and others (Riggs et al 2002). Many studies in rodent support this evidence, and recently data in humans. In fact, IL-1 or

TNF-α receptor blockers in postmenopausal women reduced the estrogen deficiency-induced increase in bone resorption by approximately 50% (Charatcharoenwitthaya et al 2007).

Cardiovascular system

The incidence of cardiovascular disease differs significantly between men and women, in part because of differences in risk factors and hormones. Indeed the incidence of atherosclerotic diseases is low in premenopausal women compared to postmenopausal women, and is reduced in postmenopausal women who receive estrogen therapy (Barrett-Connor 1997, Grady et al 1992).

Estrogens protective effects in the cardiovascular system are due to a number of beneficial activities of the hormone: they alter serum lipid concentrations, coagulation and fibrinolytic systems, antioxidant systems, and the production of other vasoactive molecules, such as nitric oxide and prostaglandins, all of which can influence the development of vascular disease.

The major estrogenic benefits seem to be connected to the direct effects on the vascular cells and tissue. Estrogen receptors α and β are expressed by endothelial cells and by smooth muscle cells and myocardium cells (Karas et al 1999, Lindner et al 1998), and their activation induces multiple effects. At physiologic concentrations, estrogen stimulates the opening of calcium-activated potassium channels through a nitric oxide and cyclic guanosine monophosphate–dependent pathway, thus relaxing smooth muscle and promoting vasodilatation (Valverde et al 1999, Wellman et al 1996). In cultured endothelial cells, hormone treatment cause a rapid release of nitric oxide, probably mediated by non-genomic mechanisms (Chen et al 1999), and of prostaglandins. Estrogen may also increase the bioavailability of nitric oxide in vessels by increasing the expression of the gene for the inducible form of nitric oxide synthase (Binko & Majewski 1998). Finally, estrogens present a clear antiproliferative activity on smooth muscle cells; since hyperproliferation and migration of this cell population

cause most of the vascular damage in atherosclerosis, this represent another protection mechanism exerted by estrogens. This vasoprotection appears to be mediated by SRC3 (steroid receptor coactivator-3); in fact transgenic mice lacking this protein, highly expressed in smooth muscle cells, exhibit pronounced neointimal proliferation and appear to be less responsive to the inhibitory effect of E2 (Yuan et al 2002).

Moreover, estrogen protection can be correlated with its ability to modulate lipoproteins serum levels. Many studies have documented that estrogen therapy in postmenopausal women decreases serum total cholesterol and low-density lipoprotein (LDL) cholesterol concentrations, increases serum high-density lipoprotein (HDL) cholesterol and triglyceride concentrations, and decreases serum Lp(a) lipoprotein concentrations (Mendelsohn 2000).

Recent discoveries show, in addition, that estrogens have the ability to inhibit lipoprotein oxidation. Have been observed indeed that near-physiologic concentrations of estradiol may inhibit the ex vivo oxidation of LDL cholesterol in plasma (Shwaery et al 1997), and that in postmenopausal women, both long-term and short-term administration of 17β-estradiol can decrease the oxidation of LDL cholesterol (Sack et al 1994).

Hepatic expression of the genes for several coagulation and fibrinolytic proteins is also regulated by estrogen through estrogen receptors. Plasma fibrinogen concentrations are decreased by continuous estrogen therapy, as are plasma concentrations of the anticoagulant proteins antithrombin III and protein S (Nabulsi et al 1993). Estrogen also decreases plasma concentrations of the antifibrinolytic protein plasminogen-activator inhibitor type 1 (Koh et al 1997), and high serum estrogen concentrations are associated with an increased overall potential for fibrinolysis.

Finally, estrogens are able to influence the key processes of atherosclerosis inflammatory traits, particularly monocytes adhesion with the endothelium, and their subsequent migration in the deepest vascular layers. The molecular basis of this phenomenon seem to involve a process in which estradiol, by means of a transcriptional mechanism, inhibit IL-1 induction of adhesion factors expressed on the

epithelium surface: VCAM-1 (Vascular Adhesion Molecule-1), E-selectin, ICAM-1 (Intracellular Adhesion Molecule-1) (White 2002).

Central Nervous System (CNS)

Traditional role of estrogen receptors in the nervous system is associated with the modulation of sexual behavior, including the regulation of gonadotropin and prolactin secretion. However, these receptors are involved also in a number of processes not associated with the reproductive activities. For instance, estrogens and androgens can influence the cognitive sphere and particularly the verbal communication, the fine motor skills, the outcome in spatial orienteering tasks and in memory tests (Hampson 1990, Sherwin 1994, Sherwin & Tulandi 1996). Moreover, they affect the coordination of fine movements in animals, and the symptoms of Parkinson and tardive dyskinesia in human subjects (Bedard et al 1977). Finally, they correlate with many depression symptoms and with the depressive disease treatment (Kendall et al 1982, Klaiber et al 1997, Regier et al 1988, Schneider et al 1997).

Is worth noticing that many estrogenic effects differ from the qualitative and quantitative point of view between two sexes; this difference is due to the sexual dimorphism established during prenatal development or during the first phases of post-natal growth. This gender difference is reflected in a different predisposition to psychiatric pathologies: depression, for instance, is much more common in female subjects (Gorman 2006), while psicoactive substances abuse, antisocial behavior and pain sensitivity appear to be common in the male counterpart (Pfaff 1980).

The complexity of this wide array of effects suggests that estrogenic actions in the brain involve many different regions of the central nervous system. Indeed estrogen receptor mapping in brain regions showed that the two ER isoforms are expressed in all the cellular type of CNS (neurons, glia, microglia, neuronal stem cells): ERβ immunoreactivity is mostly localized in olfactive bulb, cerebral cortex, septi, preoctic area, amygdale, *substantia nigra*, raphe dorsal part, *locus coerelus* and cerebellum; ERα

localization pattern appear to be close to the other isoform, even though is more concentrated in hippocampus, in preoctic area and in hypothalamus (Maggi et al 2004, Stell et al 2008).

Estrogens possess a critical role in the modulation of memory and learning processes; has been proved that treatment of menopause women with the hormone cause a benefit for the mnemonic short-term abilities, and this benefit is reversed if the treatment ceases (Sherwin 1994). In hippocampus, estrogen receptor activity is responsible for the modulation of memory processes, since it can rapidly modulate several different types of synaptic plasticity of neurons. One is spinogenesis, and another one is synaptic transmission such as long-term depression or long-term potentiation (LTD or LTP). Spinogenesis includes not only spine-synapses (spines forming synapses) but also free spines (spines without forming synapses), whereas LTD and LTP probe the characteristics of preformed synapses. Modulation of spinogenesis is essential action of estrogen in memory processes, involving production of new spines that creates sites for new neuronal contacts. Density and morphology of hippocampal spines are in fact rapidly modulated upon estradiol application, which induces a significant enhancement of the spine density, blocked by ER antagonists (Mukai et al 2006). Estradiol-induced modulation of LTD or LTP on the other hand occurs only in pre-existent synapses, because newly generated spines by estradiol treatments do not form new synapses.

Another consequence of reduced estrogens levels occurring in the second part of menstrual cycle, after pregnancy or during menopause, is a mood disturb, that comes with a depressive state. This is associated with an alteration of the seretoninergic system, involved in the mood regulation (McEwen & Alves 1999). With respect to the serotonergic system, several sexual dimorphisms have been reported and could be linked with sex differences in antidepressant response. In particular, increased 5-hydroxy-indoleacetic acid (5-HIAA) has been reported in the cerebrospinal fluid of women suffering from depression. In addition, whole brain serotonin (5-HT) synthesis and 5-HT_2 receptor binding capacity were found to be decreased in several brain

regions of women compared to men (Rubinow et al 1998). In rats, it has been shown that there is an increase in 5-HT activity, increased 5-HT synthesis and increased 5-HT metabolites in the female brain (Carlsson & Carlsson 1988, Haleem et al 1990).

Studies in animals demonstrate that reproductive steroids also regulate basal and stimulated hypothalamic-pituitary-adrenal (HPA) axis function, associated with the stress behavior. In general, low-dose, short-term administration of estradiol inhibits HPA axis responses in ovariectomized animals (Dayas et al 2000, Young et al 2001) while higher doses and longer treatment regimens enhance HPA axis reactivity to a variety of stressors (Carey et al 1995).

Different experiments suggest that both circulating and locally produced estrogens may represent a protection factor against many CNS diseases: dyslexia, autism, neurotrasmitters release alterations (depression, anorexia, bulimia), neurodegenerative diseases (Alzheimer and Parkinson diseases), traumatic pathological events (epilepsy, traumatic brain injury), immunitary system disfunctions (multiple sclerosis).

Some data seem to support the hypothesis that the protective effect of estrogen on the structural and functional brain integrity may be connected with its ability to lower the inflammatory response during neurodegenerative processes. Estrogen receptor, indeed, controls the survival of monocytes, the fagocitic components of the immunitary system, and iNOS (inducible Nitric Oxide Synthase) enzymatic activity, necessary for nitric oxide production, the main inflammatory mediator (Cuzzocrea et al 2000, Vegeto et al 1999). Other experiments focused on microglia, macrophage-like cells that are able to synthesize, when activated, inflammatory factors like nitric oxide, proteases, arachidonic acid, cytokines. The inflammatory reaction, induced with LPS in microglia primary cultures, is sharply reduced in the presence of estradiol (Vegeto et al 2001).

Liver

Estrogens play their influence in the hepatic tissue which expresses, although in lower concentration than classic target tissues, the nuclear receptors specific for the hormone.

Liver is the main site for biotransformation, conjugation and catabolism of gonadic origin hormones. It is indeed characterized by the presence of key enzymes for steroid metabolism, including aromatase.

Many data suggested that at hepatic level estrogens play a major role in cholesterol homeostasis regulation. This hypothesis comes from investigations showing that in women, before menopause, the risk associated with cardiovascular diseases is lower compared to the male counterpart of the same age; however, after menopause, when estrogen levels in the bloodstream are sharply reduced, the same risk increases significantly. Estrogen treatment in this phase causes a decrease in LDL (Low Density Lipoprotein) concentration in the bloodstream. These evidences strengthen the assumption that estradiol may have a role in cholesterol metabolism and therefore a protective effect in the pathologies associated with this molecule (Heiss et al 1980, Kuller et al 1990, Lopez et al 2002). Many liver expressed genes have been found to play important roles in controlling carbohydrate and lipid homeostasis. These genes are mainly involved in insulin signaling pathways (Schinner et al 2005), lipid and fatty acid metabolism, and glucose metabolism (Postic et al 2004), as well as cytokine signaling pathways. A further proof of this affirmation validity derives from studies in animal models estrogen-deprived: defective mice for aromatase enzyme (ArKO), or for estrogen receptors α and β. ArKO mice show obesity and hepatic steatosis within one year old; both males and females develop hypercholesterolemia, and males only have very high triglycerides levels (Jones et al 1993, Nemoto et al 2000). Knockout mice for α receptor and double knockout (for ERα and ERβ) reveal a phenotype comparable to the one observed in ArKO mice; on the other hand, no alteration of the lipidic profile have been noticed in mice lacking ERβ (Heine et al 2000, Hewitt et al 2003, Ohlsson et al 2000). In a recent study, obese (ob/ob) mice were used to investigate the effects of long-term estradiol administration on insulin sensitivity, lipidic profile and glucose tolerance on mouse liver. The results show indeed that estradiol decreased hepatic storage of triglycerides and the expression level of several enzymes involved in lipid metabolism (Gao et al 2006).

Moreover, studies in humans and rodents link the endogenous estrogen hormone to the maintenance of glucose homeostasis. Indeed, postmenopausal therapy with estrogen may reduce the incidence of type 2 diabetes and that treatment of healthy postmenopausal women with unopposed estradiol or conjugated equine estrogen has been shown to improve insulin sensitivity and to lower blood glucose (Bonds et al 2006, Espeland et al 1998, Saglam et al 2002). These indications found support from data collected in type 2 diabetes rodent models, such as db/db mice and Zucker diabetic fatty rats, in which male rodents develop hyperglycemia, whereas female rodents are protected (Louet et al 2004). Again, studies in ERα and ERβ knockout mice have demonstrated that ERα, but not ERβ gene depletion, results in increased body weight, glucose intolerance, and insulin resistance (Heine et al 2000).

Lungs

Although only recently lungs gained attention as a possible target of ovarian hormone action, many studies confirmed the hypothesis of a pivotal role of estrogens in this organ development and homeostasis.

The reason why lung estrogenic effects have long been ignored might be correlated with the fact that ERα, the first discovered and best known receptor isoform, is missing in the pulmonary tissue, which expresses instead the isoform β, identified in 1996 (Kuiper et al 1996). The significance of the role of this isoform in the lung was demonstrated again by the observation of knockout mice lacking ERβ: they are characterized by noticeable structural and functional alterations, and by deep biochemical alterations of alveolar epithelial cells. Female adult mice reveal a deposit of surfactant and defects in the alveolar development, causing a sharp decrease in alveoli number (Patrone et al 2003). Key factors mediating the regulation of alveoli development and homeostasis are: platelet-derived growth factor A (PDGF-A) and granulocyte macrophage colony-stimulating factor (GM-CSF). Their level is clearly reduced in females deprived of ERβ, demonstrating a direct transcriptional regulation

exerted by estrogen via ERβ (Patrone et al 2003). GM-CSF is a soluble proteic factor that play a primary role in the lung development and homeostasis regulation: it is indeed a growth factor necessary for bronchiolar cells and for alveolar type II cells (Reed & Whitsett 1998); this gene deletion deeply weakens the alveolar functionality, leading to the surfactant deposits (Dranoff et al 1994, Robb et al 1995).

These results provide some basis for a possible model of the molecular mechanism leading to the gender differences observed in the alveolar structure of adult lungs; ERβ appears to be a key regulator of pulmonary development and homeostasis after birth.

Many physiological and pathological aspects are indeed sexually dimorphic, both during development and in the adult. Lung maturation, for instance, is more rapid in the female counterpart compared to the male during the fetal development; this is probably due to the androgen inhibitory effect and to the estrogen stimulatory effect [Ballard, 1989]. Moreover, women are significantly more prone to develop lung obstructive diseases associated with cigarette smoke and lung cancer, especially denocarcinoma, a histological subtype that correlates with worsened prognosis (Connolly et al 1991, Coscio & Garst 2006, Sekine et al 1999, Zang & Wynder 1996). Epidemiologic evidence from a number of studies suggests that women are more susceptible to tobacco-induced carcinogenesis than men, taking into account baseline exposure, body weight, body height, and body mass index (Stabile & Siegfried 2003).

Immune system

Several experimental studies suggest that sex steroids influence immune cell development in primary lymphoid tissues (bone marrow and thymus) and, in addition, have immunomodulatory effects on both peripheral T cell and B cell subsets in adult life. The two estrogen receptor isoforms are expressed in a number of immune tissues and cells; particularly, ERα has been identified in bone marrow, and both isoforms are expressed in thymus.

A wide array of evidences suggests the hormonal influence on every lymphoid tissue; among them, thymus appears to be the most responsive one. thymus expresses both ERα and ERβ and normal thymic development is dependent on the estrogenic signaling pathway, as shown by reduced thymic size in neonatal mice lacking ER. Despite the involvement of ERα signaling in normal thymic development, administration of exogenous estrogen to pubertal or adult animals induces thymic atrophy and immune suppression. However, early estrogen exposure during the perinatal period can induce either decreases or increases in thymic weight, and potentially, functionality; this is variable depending upon the age of the animal and dose (Erlandsson et al 2001, Forsberg 1996, Yellayi et al 2000).

Moreover, these hormones regulate T-cells mediated immune functions: mice treated with estrogens or testosterone show a decrease in ornithine-decarboxylase (ODC) activity following mitogenic stimulation; indeed estrogen treatment cause depletion of CD4 and CD8 positive cells (Screpanti et al 1989). Sexual hormones alter epithelial thymic cells ability to release different factors necessary for the intrathymic differentiation of T-cells (Vegeto et al 2003). In addition, they can influence IL-6 and IL-7 production from thymus stromal cells.

Natural-killer cells (NK cells) generated in the bone marrow are another target of estrogen action in the immune system. These cells are considered as part of the primordial immune system, and contribute to the elimination of exogenous pathological agents with cytotoxic mechanisms, neutralizing virus infected cells, lysing some bacteria and regulating hematopoiesis. The ER pathway reduces their activity, both in normal mice and in mice with autoimmune diseases. On the other hand, sexual male hormone seem to have a minimal influence on these cells (Kalland 1980, Kalland 1984, Pan et al 1986).

Finally, have been shown estrogen involvement in seric and uterine immunoglobulin synthesis regulation, including IgM, IgA and IgG (Ansar Ahmed et al 1985). It has been know for long time estradiol role in increasing antibodies production, directed against a wide array of antigens, including autoantigens (Ansar Ahmed et al 1989). This

suggests that even T-cells may be target of hormone action, even though a direct effect on these cells is still to be confirmed.

Lastly, estrogens play a role in regulating phagocytic cells fate. Female rats count more adrenal macrophages compared to the male counterpart; male rats treated with estradiol indeed show a peak on the macrophages number, not detected in control animals (Magalhaes & Magalhaes 1984).

4. An animal model for the study of ER activity: the ERE-Luc mouse

The necessity to monitor the estrogen receptor transcriptional activity in the animal, in physiological conditions or following pharmacological treatment, led our laboratory to develop a transgenic mouse model where the reporter gene, firefly luciferase in this particular case, was expressed ubiquitously in every animal tissue and was under ER transcriptional control.

The generated model therefore provides a quantitative parameter for the receptor activity evaluation in each target organ, thanks to biochemical, immunohistochemical and *in vivo* imaging combined approaches. The key feature of this model is the ability to represent a physiological condition, mirroring the real action of the receptor modulation in vivo, with no direct modifications to the estrogenic signal, such as the introduction of ERα and ERβ mutations.

The generation of this model opens up new important horizons for the pharmacological research related to the possibility to carry out toxicological, pharmacokinetics and pharmacodinamics studies in a rapid, effective and simple way.

Moreover, imaging *in vivo* tools allow to obtain a global vision of the tissue-specific effects of estrogenic compounds, and to predict potential adverse effects already in the preclinical studies first phases.

The initial problem related to the development of such a system was to obtain an ubiquitous and adaptable transgene expression, in order to achieve an active biosensor in every target tissue. Since in our case the transgene integration is not leaded by homolog recombination events, it can occur casually in every genomic region. It is well known that the expression of transgenes regulated by weak promoters is highly influenced by transcriptional regulatory elements (enhancers/silencers) localized nearby the integration site. Moreover, some genomic modifications, such as acetylation

and methylation, may negatively influence the levels of transgenic expression. Is worth remember that in every cell, based upon the differentiative state, the genetic material is existing in two different forms: euchromatin, the relaxed form, accessible to the transcriptional machinery, and heterochromatin, the condensed and inactive form, not to be transcribed. If the transgene is integrated in the heterochromatinic zone, the expression will be compromised. All these effects, defined "positional effects", lead to an inappropriate transgene regulation. However, some sequences called insulators, proposed as elements able to reduce the positional effects, can allow the transgene isolation from the chromatinic context (Bell & Felsenfeld 1999). Insulators were first described in the 80's, when was observed that they are able to increase transgenic expression in single specific tissues (Sun & Elgin 1999); the use of these sequences, placed in the 3' and 5' ends of the transgene, establishes a permissive domain in the genome allowing the transgene expression, and creates a barrier against regulatory sequences influence and against methylation and acethylation events. For the generation of the transgenic model were used MAR (Matrix Attachment Regions) insulator sequences [Stief, 1989], derived from chicken lysozyme, and HS4 (Hypersensitive Site 4), derived from β-globine gene (Chung et al 1993).

Another relevant issue in this model generation was the reporter gene choice. The final decision to use firefly luciferase gene was driven by four reasons:

- This genes codify for an enzyme that is present in insects and absent in mammals, allowing minimizing the endogenous background;
- To date, in mice have not been described luciferase structurally similar proteins; this reduces the chance to have aspecific signals, derived from antibody cross-reactivity phenomena in immunohistochemitry analysis.
- The assay for the quantitative detection of luciferase enzymatic activity in tissue homogenized is highly sensitive and reproducible.
- Enzyme half-life in mammal cells is short, therefore we failed to observe luciferase deposit in target cells, allowing a dynamic detection of the receptor activity (Thompson et al 1991).

In order to obtain a reporter system assuring a basal expression level and a powerful induction ability following receptor activation were tested different estrogen-responsive promoter compositions, using transfection assays in carcinoma mammary cells MCF-7, in neuroblastoma SK-N-BE, and in uterine carcinoma HeLa. The tested constructs included deletion mutants of the minimal tk (Thymidine Kinase) promoter of HSV (Herpes Simplex Virus), combined with multimeric estrogen responsive sequences ERE. The most effective combination was generated with two ERE palindromic sequences, separated by 8 base pairs and localized 55 nucleotides upstream the tk promoter.

The reporter transgene was flanked by insulator sequences, tested with stable transfection assays in MCF-7 cells, to verify the real expression independency from the chromatinic context. These experiments showed that transgene expression in pMAR or pHS4 constructs is more frequent than pERE construct lacking insulator sequences; moreover, these elements allow a regulated transgene expression.

The linearized vectors were then injected in oocytes derived from C56BL/6x DBA/2 second generation mice. This outbred strain was chosen to increase the transgenic efficiency; moreover, C56BL/6 presence in the genetic background confer a good estrogenic responsivity (Roper et al 1999, Spearow et al 1999).

From twelve generated lines, three showed an inducible E2-expression of luciferase. From these lines, the one showing a better ubiquitous expression was expanded and characterized, to verify the transgene inducibility in every target organ. The hormonal treatment cause at least a five-fold increase in the enzyme expression compared with non treated control animals, in tissues such as liver, lung, spleen, bone marrow, brain, thymus. In uterus, bladder, skin, adipose the same treatment determine an increase between 2.5 and 4.9 fold compared to controls. On the other hand, hearth, pancreas, blood, tail, aorta, esophagus, thyroid, stomach, tongue and skeletal muscle failed to show, in this line, any responsivity to estrogenic stimuli (Ciana et al 2001).

From the confrontation of the tissue distribution of ERα and ERβ receptors and luciferase distribution, a close correlation emerges between the hormonal treatment responsivity and the presence of the receptor.

Another proof of the model validity was obtained through himmunohistochemistry experiments, which allowed detecting a colocalization of luciferase and estrogen receptor in stroma, endometrium and gland epithelium in ovariectomized mice female uteri treated with estradiol.

5. Aim of the study

In recent years, the ERE-Luc reporter mouse allowed us to follow the real-time activity of Estrogen Receptors directly interacting with ERE-like binding sites in a number of physiological and pathological conditions and in *in vivo* animals. By the analysis of these activation patterns in intact and cycling females, have been observed that the receptor activity oscillates in different organs; this pattern appears to be independent from the circadian pattern, and shows a regular trend with a frequency of 4,5 days. This regular ciclicity may be associated with the hormonal levels observed in intact female mice during oestrus cycle. Liver estrogen receptors, particularly, seem to activate in synchrony with serum estrogenic levels, indicating a possible estrogenic implication in the hepatic functions.

A growing body of evidences suggest that lipidic and glucidic metabolism in liver tissue might be regulated by estrogenic action, as indicated in studies on diabetic ob/ob mouse models (Gao et al 2006), or as suggested by the deregulation of the lipidic profile during menopause. Moreover, genome-wide identification of estrogen receptor binding sites in liver tissue of ovariectomized mice estradiol-treated highlighted that genes that recruited ER to their promoters belong to the same gene ontology categories involved in lipid metabolism, such as lipid biosynthesis (Gao et al 2008). However, little is known about the role of estrogen in the control of the liver metabolic functions in physiological conditions, and about the hepatic genes that are regulated in the healthy mature female organism.

For this reason we decided to focus our investigations on adult cycling female mice, to check whether gene expression and estrogen receptor binding to chromatin may be influenced by the subtle but persistent in time changes in circulating estradiol concentrations.

Our final goal is to characterize and frame the physiological estrogen-dependent expression profile, and to compare it with the profile in other physiological conditions, such as prepuberty, pregnancy and, importantly, menopause.

6. Methods

Animal maintenance

Mice were maintained in a 12-hour light-dark cycle and housed one to four per cage with food and water *ad libitum*. Wild-type mice, used to generate the transgenic lineages, are C57BL/6 background (Charles River Laboratories).

Pups are separated from the mating parents and between males and females when they reach 21 days. Ear punches, a procedure necessary to mark and discriminate individual mice in the same cage, is done on animals 4-weeks old. Is obtained creating a small (0.5 to 2 mm) notch with a punch device (National Band & Tag Co., USA) near the edge or in the middle of the ear; the notch would be in different and ordered positions, following a unique and progressive code. Tail tipping is the following step if genotyping is necessary.

Evaluation of the estrous cycle phase

Vaginal smear

Vaginal smear is a technique commonly used to assess the estrous cycle phase in mature and intact female mice. Animals are grasped firmly from the back, rotated with the abdominal portion upwards to expose the vaginal lining. A small amount (approximately 0.2 ml) of saline or distilled water is drawn up into the pipette tip. The tip of the pipette is pushed gently into the entrance of the vagina to a depth of 2-5 mm and the fluid is flushed into the vagina and back up into the pipette two or three times by gently squeezing and releasing the bulb of the pipette. If the fluid is seen to be 'cloudy' after the first flushing the subsequent flushings are unnecessary. A small amount of the cell suspension is then expelled onto a labelled glass slide. Slides should

be labelled with the female identification numbers. The tip of the pipette should be rested on the slide at an angle when placing the sample on the slide, to maximise control of the volume and prevent contamination from splashes, which may occur if drops of the sample are expelled from a height. Up to two smears may be placed on a single slide and they are air-dried.

Smear staining

For the smear staining a commercial kit is used (MGG Quick stain, Bio Optica), derived from the May Grunwald-Giemsa technique. This is a quick method for differential staining of formed blood elements and cytological preparations.

Based on three reagents:

A - Fixative

B - Eosine solution

C - Thiazine staining solution

Glass slides are immersed 5 times for 1 second each in solution A (fixative), then solution B (Eosine solution) and finally solution C (Thiazine staining solution), ensuring to wait a moment after every immersion to drain liquid excess. Procedure is completed washing the slides with tap or distilled water and drying the slides in the air.

Microscope analysis

Air dried vaginal smears on glass slides are eventually observed with an optical microscope (Axioskop 2 MOT plus, Zeiss) using a final magnification of 100X.

The analysis for the identification of the estrous cycle phase is based on the assessment of the cytological appearance of the smear. The four basic stages of the cycle (estrus, metestrus, diestrus and proestrus) can be recognised by the presence, absence or proportional numbers of epithelial cells (two types), cornified (keratinised) cells and leucocytes. Occasionally mucus is also seen, especially in acyclic females. Epithelial

cells seen at proestrus are mostly nucleated and with a granular appearance; those seen at metestrus tend to be non-nucleated and less granular.
It is the balance of proportions between these cell types that permits the classification of the stage of the cycle between successive ovulations.

Mice anesthesia

The anesthetic solution used to achieve a medium to long-lasting anesthesia of the animal is based on ketamine (Ketavet 100, injectable solution, Gellini) and 50 mg/ml and xilazine (Rompum, Bayer) 20 mg/ml. The final dose of the treatment is ketamine 50 mg/Kg + xilazine 20 mg/Kg.
A 0.5 ml syringe (Becton & Dickinson) is loaded with the necessary volume of anesthetic solution, usually between 50 and 70 μl/animals (depending on the body weight). The mice are immobilized by the scruff method, and the needle is inserted parallel to the skin and directed toward the posterior of the animal. When the injection is properly done, the animal is placed back in the cage, kept in the dark. Wait at least 10 minutes before any other manipulation to allow the distribution of the solution.

Ovariectomy

This is a procedure resulting in the removal of the ovaries, the main source of estrogen in the female mice. The animal is prepared for surgery using the pre-operative anesthetic procedures as described above. Confirm that the animal shows a reduced respiratory rate and no response to gentle pinching of foot pad. Both flank of the animal should be shaved and the shaved is then swabbed with 70% ethanol. All instruments should be sterilized by dipping in 90% ethanol and then flaming in a Bunsen Burner or by other accepted methods of sterilization.

A 5mm, dorsal/ventral incision is made through the skin of the flank of the mouse below the muscles surrounding the spinal cord. The incision is centred between the bottom of the rib cage and the front of the hind limb. In the rat, a 10mm incision is placed in a similar fashion. The skin is separated from the underlying muscle.

Before making the incision through the muscle overlying the ovary, confirm the location of the ovarian fat pad which is sometimes visible under the muscle. Rather than cutting the muscle, insert the tip of double sharp iridectomy scissors just through the muscle layer, and separate the muscle fibers by opening the scissors in a dorsal ventral direction. Hold the edge of the incision open with a small rat tooth forceps and pull the ovary through the incision with a blunt forceps by grasping the fat pad surrounding it. Place a mosquito haemostat at the boundary between the oviduct and uterus, and place a ligature just below the haemostat. After removing the ovary and oviduct with a scissors, release the haemostat and make sure no bleeding occurs. Return the ovary to the abdominal cavity, and suture the muscle layer if necessary. Close the skin incision with wound clips. Turn the animal over and repeat the procedure on the other side.

Return the animal to its cage and leave undisturbed in a warm, quiet place. Monitor the animal continually until it is completely recovered from anaesthesia. If clear signs of pain, acute discomfort, or adverse reaction to the drug are apparent (e.g., convulsions, respiratory distress), the animal should be euthanized. Following recovery, the animal should be monitored daily for one week for signs of infection or persistent problems, in which case the animal should be euthanized.

Animal sacrifice and organ collection

Mice sacrifice is done after complete anaesthesia with cervical dislocation.

Organs of interest are harvested using sterilized chirurgical instruments. Once collected, organs are immediately snap-frozen submerging them in liquid nitrogen or

placing them on numbered aluminium pieces over a dry-ice surface. When frozen, they are kept at -80°C until the subsequent analysis.

Blood and serum collection

The technique used to collect blood from mice allowing them to survive is the retro-orbital sinus blood collection. The retro-orbital sinus is the site located behind the eye at the medial or lateral canthus.

Anesthetize the mouse and lay it down on its side. The skin above and below the eye should be pulled away from the eyeball, so that the eyeball is protruding out of the socket as much as possible. Insert the tip of a fine-walled Pasteur pipette or a microhematocrit blood tube into the corner of the eye socket underneath the eyeball, directing the tip at a 45-degree angle toward the middle of the eye socket. Rotate the pipette between your fingers during forward passage; do not move it from side to side or front to back. Apply gentle downward pressure and then release until the vein is broken and blood is visualized entering the pipette. When a small amount of blood begins filling the pipette, withdraw slightly and allow the pipette to fill. Then pour the blood into a 1.5 ml plastic tube kept in ice, repeat if necessary. When finished, close the plastic tube and spin in a cold (4°C) centrifuge for 10 minutes at 8000 rpm. Transfer the supernatant (serum) in a new tube and store at -80°C.

Bioluminescence Imaging (BLI)

In vivo BLI

Briefly, a CCD consists of a sensor for recording images, based on an integrated circuit containing an array of linked, or coupled, capacitors. Under the control of an external circuit, each capacitor can transfer its electric charge to one or other of its neighbours enabling to generate a digital image based on optical and UV spectroscopy. In principle,

the CCD enables also to quantify the photons emitted by selected areas of a living animal. Under anaesthesia, 20 minutes before BLI, animals were administered i.p. 50 μL of a water solution of the luciferase substrate luciferin (Beetle luciferin potassium salt, Promega, Madison, WI, USA) corresponding to 50 mg/kg for a 25 g mouse. Bioluminescence was measured by a Night Owl imaging unit (Berthold Technologies) consisting of a Peltier cooled charge-coupled device slow-scan camera equipped with a 25 mm/f 0.95 lens. Background was estimated on the average of ten background acquisitions and arithmetically subtracted from the raw images. Before each imaging session, instrumental efficiency was measured with an external source of photons (Glowell, Lux Biotechnology, Edinburgh, UK). For photon emission measurement, mice were placed in a light-tight chamber, a gray-scale image were first taken with dimmed light, then luciferase signal was registered for 5 minutes. Anatomical areas (limb and tail/skeletal, genital, hepatic, abdominal and thymic) were defined and in each anatomical area, photon emission was calculated as the number of counts per second per centimeter square (cts/cm2/s) corrected for instrument efficiency. All the measurements were in the linearity range of the detector.

Luciferase enzymatic assay

For luciferase enzymatic assay, tissues were homogenized in 500 μl of ice-cold lysis buffer (100 mM KPO4,1 mM DTT, 4 mM EGTA, 4 mM EDTA, pH 7.8) with a 5 mm inox bead in a Tissuelyser (Qiagen), undergone one freezing-thawing cycle, and were centrifuged for 30 minutes at 4900 x g, 4°C (Rotanta 460R Hettich Zentrifugen). Supernatants containing luciferase were collected and protein concentrations measured by Bradford assay, following reagent's manufacturer instructions (Pierce Biotech). Luciferase enzymatic activity was assessed by mixing 20 uL of tissue extracts (diluted 1:15 to prevent matrix interference) with 100 uL of a commercial luciferase assay buffer (Promega). Light intensity was measured with a luminometer (Glomax, Promega) and expressed as relative light units over 10 sec/μg protein (RLU/μg prot).

Expression microarrays

RNA extraction and retrotranscription

After liver harvesting, 30 mg of frozen liver tissue were weighted and used for RNA extraction with a commercial kit (RNeasy, Qiagen) following the indications in the protocol. The tissue was homogenized in RLT buffer with TissueLyser machine (Qiagen). RNA extracted was quantified using Nanodrop Spectrophotometer.
1 μg RNA was then retrotranscribed to cDNA using random primers and MMLV retrotranscriptase.

Microarrays

For the expression analysis we used GeneChip Mouse Genome 430 2.0 Array (Affymetrix), with more than 45,000 probe sets able to analyze the expression level of over 39,000 transcripts and variants from over 34,000 well characterized mouse genes.

Microarray data analysis

Analysis of the hybridization intensities reported was done with dChip software (a free software started in Wing Wong Lab and developed and maintained by Cheng Li Lab - Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Harvard School of Public Health, Boston, MA, USA).

Chromatin Immunoprecipitation on Tiling Arrays (ChIP-on-chip)

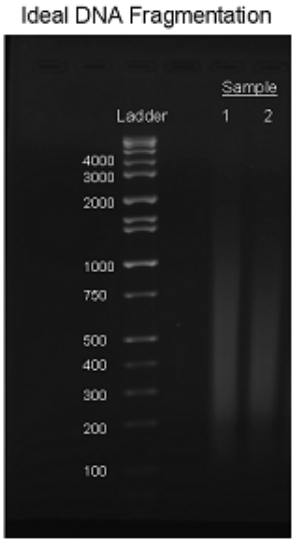
ChIP-chip is a technique that combines chromatin immunoprecipitation and microarrays to identify in vivo TF-binding sites and protein-DNA interactions in a

high-throughput manner. Briefly, DNA and protein are cross-linked in vivo with formaldehyde treatment and immunoprecipitated with specific antibodies against the protein of interest. DNA bound by protein is fragmented by sonication. Finally, the pulled-down DNA and appropriate controls are labeled and applied to microscopic slides for microarray analysis.

Chromatin Immunoprecipitation (liver tissue)

After mouse sacrifice, whole liver is harvested and placed immediately in 1 ml of 37°C Dulbeccos PBS until ready for fixation. Majority of Dulbecco's solution is then removed, and the tissue, chopped in tiny pieces to allow better crosslinking, is transferred to pre-warmed 1% formaldehyde solution in 50 ml conical tubes. The tube is left in the warm room (37°C) shaker for 15 minutes. After the crosslinking step is done, aspirate crosslinking solution and wash 1 time with cold 5mg/ml BSA/PBS; shake gently, allow tissue pieces to settle and aspirate supernatant. Repeat the washing with 10mls ice-cold PBS, and resuspend the samples in PBS enriched with proteinase inhibitors, to avoid protein degradation during the process. The sample is therefore moved to a pre-chilled dounce homogenizer and homogenized with 10-15 strokes or until no visible chunks of material remain. Transfer the homogenate to a 2ml plastic tube and spin 1 minute at 2000 rpm in the cold (4°C) centrifuge and discard the supernatant and keep the pellet. After the lysis buffer (1% SDS, 10mM EDTA, 50mM TRIS-HCl pH 8.1 + Protease Inhibitors, made fresh) is added to the pellet, samples are ready for sonication that will shear the cellular membranes and the DNA in small fragments. Volumes of lysis buffer must be determined in relation to the amount of tissue present. Protein extract should not be too dilute to avoid loss of protein and to minimize the volume of samples to be loaded onto gels. The minimum concentration is 0.1 mg/ml; optimal concentration is 1-5 mg/ml. Sonication is performed in polystyrene tubes with tip sonicator with the following conditions: 20% amplitude, 10 times 10 seconds each, 30 or more seconds pause on ice. Sonication efficiency can be checked by running 100-500 ng of purified

DNA sample on an agarose gel; typically, sheared DNA size ranges from 100-4000 bp, with the average size fragment between 200-1000 bp (figure).



After this step, spin 15 min at 13.500 rpm at 4C and keep the supernatant. This is diluted 1:5 to 1:10 in dilution buffer (1% Triton X-100, 2mM EDTA, 150 mM NaCl, 20mM Tris-HCl pH8.1) and added to A/G Dynal magnetic beads prebound to antibody of choice (Estrogen Receptor antibody MC-20, Santa Cruz Biotechnology). Incubate the sample with the antibody between 4 hour to overnight (depending again on the amount of protein and affinity properties of the antibody), at 4°C, preferably under agitation. When the incubation time is over, centrifuge the tubes, collect the beads with the magnetic collector, remove the supernatant and wash the beads in RIPA buffer (50mM HEPES pH7.6, 1mMEDTA, 0.7%NaDeoxycholate, 1% NP40, 0.5M LiCl) three times, incubating on rotator for 10 min at 4°C between every wash. Finally wash two times with TE pH 7.5 and proceed with the reverse crosslinking step: add 100 μl Elution Buffer and place in 65°C water bath; vortex beads in this solution every few minutes for the first 30 minutes then leave at 65°C for a minimum of 8 hours or overnight. Then purify the DNA with Qiaquick Spin Kit.

Fragment amplification

Fragments obtained by the chromatin immunoprecipitation process are not sufficient to be hybridized on the chip. Therefore fragment amplification is a mandatory step to obtain enough material to be spotted on the slides.

RNase and Proteinase K treatment

This step will remove all the remaining RNA fragments and proteins from the sample. Collect between 1 and 2 ng of total DNA, bring volume to 200 µl in TE pH 7.5 and add RNase (Sigma) to a final concentration of 0.2 µg/µl. Incubate at 37°C for 1-2 hours. Then add proteinase K (Roche) to a final concentration of 0.2 µg/µl and incubate at 55°C for 2 hours. Eventually extract with an equal volume (200µl) of phenol:chloroform:IAA and transfer top layer to new tube. Include in the mix: 30ug of glycogen (Invitrogen), 1:10 volume of 3M NaOAc pH 5.2, 2 volumes of 100% EtOH and incubate 30 minutes, placing in -80°C freezer or on dry ice. After spinning max speed for 15 min at 4°C, remove supernatant and wash with 150 µl of 70% EtOH. Air dry the pellet and then resuspend in 17 µl of distilled water.

End Filling and blunt ended ligation

For the blunt end, add to the sample, on ice, the following reagents:

- 2.5 µl 10x End-it buffer
- 2.5 µl End-it ATP (10mM)
- 2.5 µl End-it dNTP (2.5mM)
- 0.5 µl End-it Enzyme Mix

And incubate 30 minutes at room temperature.

Add 75 µl dH_2O to bring up the volume and then extract with phenol:chloroform:IAA as described above.

When the pellet is dry, add 3.3 µl distilled water and allow the sample to completely resuspend. Therefore linkers must be added to the blunted end: on ice, add the following reagents to the DNA:

- 2X NEB quick ligase buffer
- Annealed linkers (15 µM)
- Quick DNA ligase (NEB)

And incubate for 5 min at room temperature, and then leave at 16°C overnight. Add 80 µl H_2O to bring up volume and extract in EtOH as described above. Eventually resuspend dried pellet in 25 µl of dH_2O.

Ligation Mediated-PCR

To 25 µl of samples add 25 µl of the following reagents:

- 5 µl 10X Thermopol buffer (NEB)
- 1.25 µl 10.0 mM dNTP mix
- 1.25 µl of 40 µM of the longer linker
- 1 µl Amplitaq (Applied Biosystems)

And run the following program in the thermocycler:

55°C 4 min

72°C 3 min

95°C 2 min

95oC 30 sec

60°C 30 sec

72°C 1 min

go to step 4, 20 more times

72°C 4 min

4°C forever

Clean up with Qiagen PCR Purification kit and elute in 50 µl buffer EB (elution buffer). For the whole human and mouse genome arrays at least 14 µg total of DNA of good quality are required.

Labelling

DNA sequences immunoprecipitated and amplified must be fragmented to 50-100 bp and labeled with biotin before the hybridization on the array.

Fragmentation is obtained with DNAse I (Affymetrix) diluted 1:31 in 10mM Tris pH 7.8 in nuclease-free H_2O and running the following program on the termocycler:

37°C 30 min

95°C 15 min

4°C hold

Good fragmentation is checked on agarose gel.

TdT buffer and biotin are added to the DNA, and left for 16 hours at 37°C:

37°C 16 hours

95°C 10 min

4°C hold

The samples are then ready for hybridization.

Hybridization

The array used was the GeneChip® Mouse Tiling 2.0R Array Set (Affymetrix); this is a seven-array set with sequences selected from NCBI mouse genome assembly (Build 33). Oligonucleotide probes are synthesized in situ complementary to each corresponding sequence. Probes are tiled at an average resolution of 35 base pairs, as measured from the central position of adjacent 25-mer oligos, leaving a gap of approximately 10 base pairs between probes. Each array in the set contains over 6.5 million perfect match probes to specifically interrogate genomic regions.

ChIP-on-chip Data Analysis

Data analysis was performed using MAT (Model-based Analysis of Tiling arrays) algorithms. Statistical significance of the array hits was set on pVal<$10e^{-5}$.

Quantitative PCR analysis

Quantitative real time polymerase chain reaction (Q-PCR/qPCR/qrt-PCR) or Real-Time PCR was used to quantify the relative expression of genes of interest in different conditions. For the detection, SYBR Green technology was used, with 36B4 as reference (housekeeping) gene.

After retrotranscription step (1 μg RNA in 25 μl), cDNA was used for the PCR amplification, using SYBR green mastermix.

7. Results

Preparation of samples

Liver ER activation correlates with circulating estradiol

Three months-old female ERE-Luc mice were followed for three weeks, checking daily their estrous cycle phase with vaginal smear technique (see Materials and Methods). Vaginal smears were done at early morning (9:00 am) and were followed by a 50 μL of an i.p. injection of a water solution of the luciferase substrate luciferin; after 20 minutes, the animals photon emission was acquired with a CCD camera device. In this way, we were able to associate the level of Estrogen Receptor activity in mouse body areas with the estrous cycle phase, and therefore with the estrogenic level of the mice.

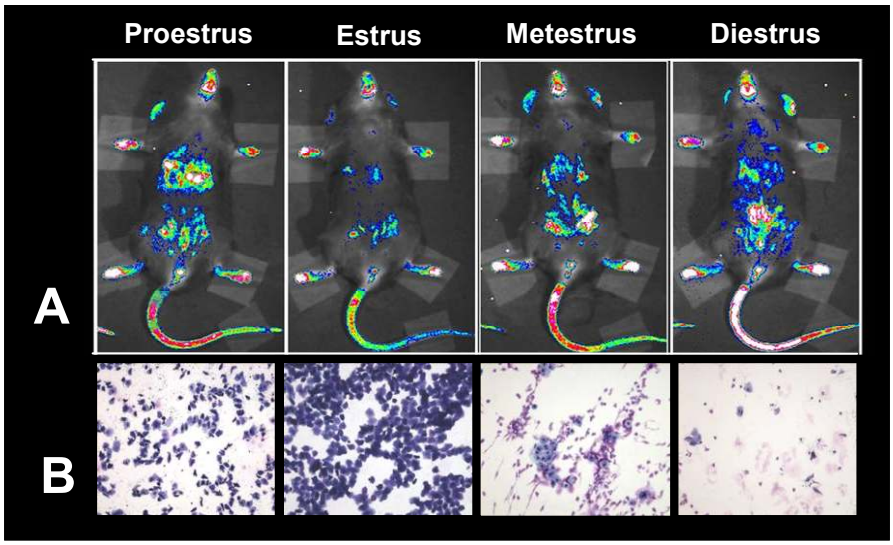


Figure 4 - Physiological changes observed during the estrous cycle. **A.** Optical imaging of the bioluminescence emitted from a representative living ERE-luc mouse during the four phases of the estrus cycle (Proestrus, Estrus, Metestrus, Diestrus). Images in pseudocolor report the level of activity of Estrogen Receptor in the mouse body areas. **B**. Representative images of vaginal smear morphological features during the four estrous cycle. Images were acquired with Zeiss Microscope at 100X magnitude.

In this analysis, we can observe that hepatic area shows a definite activation pattern, that seems to follow the levels of hormones in the bloodstream: at proestrus, when

there is a peak of circulating estradiol, ER activity in liver appears to be higher than in the following phases (estrus, metestrus, diestrus) associated with a decrease in estrogen levels.

To better assess this connection between estradiol and ER activation in the liver, we used two approaches: first, we collected serum samples from female mice at the selected estrous cycle phases; at the same time points, also liver tissue was collected.

Serum samples were used to detect the level of the sexual hormone in the blood. As expected, we measured a peak of estradiol in the proestrus phase, and lower levels in the others. In parallel, we assessed the Estrogen Receptor activity in hepatic tissue with the enzymatic luciferase assay (fig. 4). Data suggest a strong correlation between estradiol levels and level of activation in the liver of these mice.

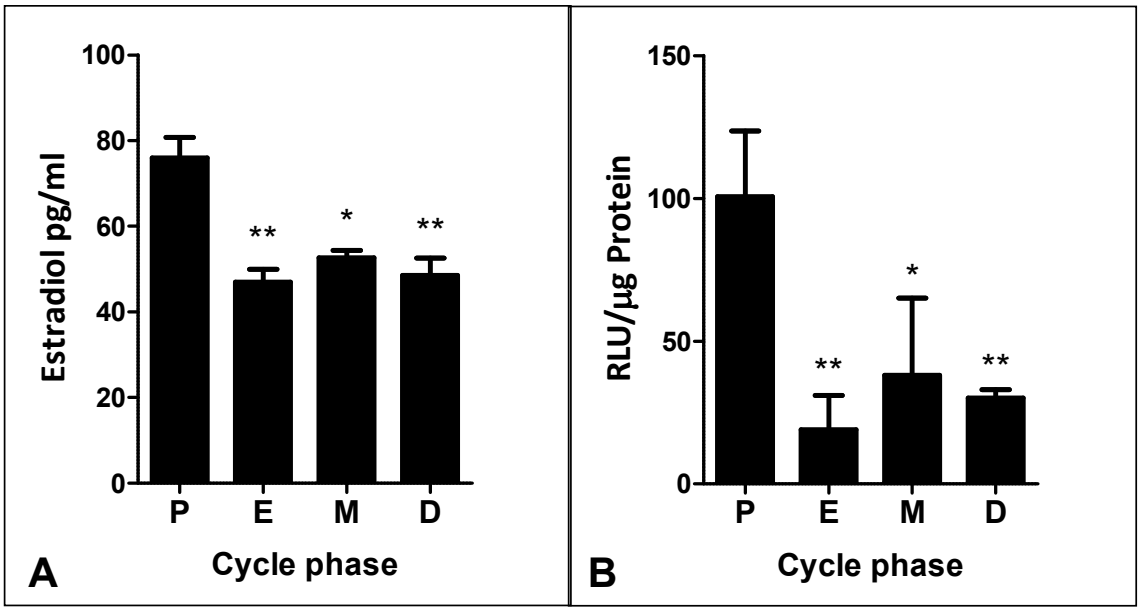


Figure 5 - A. Serum levels of estradiol, reported as pg/ml, detected in the four phases of the estrous cycle in intact female animals. Analysis of hormone levels was executed in collaboration with Dr. AF Parlow, Harbor-UCLA Medical Center (Torrence, CA, USA). Serum collection was carried out as described in Material and Methods, for three animals each phase. Data are shown as mean with SAM; statistic significance calculated with One-way Anova with Bonferroni Bost-test: *, 0,05>Pval>0,01 compared to Proestrus; **, 0,01>Pval>0,001 compared to Proestrus B. Quantification of luciferase emission from hepatic tissue calculated as RLU (Relative Light Units) and normalized over the amount (ug) of proteins in the sample. Livers were harvested and a Luciferase enzimatic assay was carried out as described in Material and Methods. Statistic significance calculated with One-way Anova with Bonferroni Bost-test: *, 0,05>Pval>0,01 compared to Proestrus; **, 0,01>Pval>0,001 compared to Proestrus

Basing on the results obtained, for subsequent analysis animals were selected to fill two experimental groups: proestrus and metestrus. The first one represents a condition with high circulating estrogens; on the other hand, metestrus represents a condition with the lowest level of sex hormones in the bloodstream.

Microarray Analysis

Hepatic tissue samples of the two experimental groups were used for the RNA extraction as described in Materials and Methods section.

Livers were harvested and RNA, after extraction and quantification, was retrotranscribed to cDNA. cDNA of the two samples were labelled with red-fluorescent and green-fluorescent dye, respectively, mixed and competitively hybridized to the microarray containing the complementary probes of almost 40,000 known transcripts.

Using a laser scanner, TIFF images of the microarray are obtained. The relative abundance of one or the other sample is represented by a red or green signal at the spot location. Must be performed therefore a microarray image analysis to quantify the spot intensities.

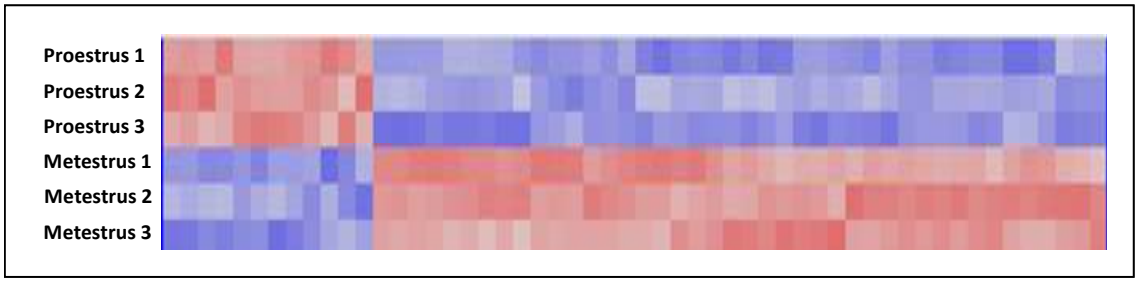


Figure 6 - Hierarchical clustering of data from the dChip analysis of gene expression. In the heatmap, red represents high expression, blue represents low expression. Only genes with a significant different expression are shown (pVal<0.05)

Once obtained the numeric data related to the spot intensities, these values must be normalized and analyzed to identify the statistically significant differences in signal. If

the spot intensities in the two conditions that have been compared are statistically different, that means that the expression of the genes associated with the probes spotted in those microarray areas is significantly different.

From the analysis of the spot intensities with dChip software we identified nearly 60 genes differentially expressed from proestrus phase to metestrus phase. Particularly, we recognized an important group of genes that appear upregulated in metestrus phase, and downregulated in proestrus phase. On the other hand, genes upregulated in proestrus represent the minority of the identified genes.

Validation of gene expression results

Quantitative real-time PCR (qPCR) is a commonly used validation tool for confirming gene expression results obtained from microarray analysis; this is because qPCR is a more precise and sensitive technology compared to microarray. Therefore we selected the most expressed genes, and we measured their expression in the two conditions with real-time analysis.

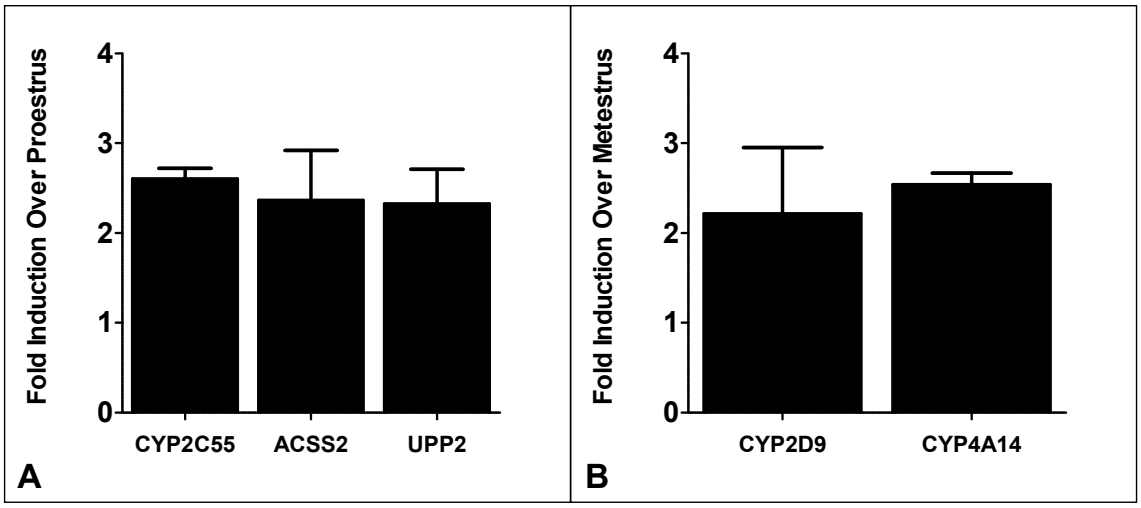


Figure 7 - Validation of the expression of selected genes identified with the microarray analysis. In panel A is; in panel B is shown shown the expression of the genes appearing upregulated in proestrus, as fold induction over the expression in metestrus. Error bars represent standard error SEM.

The genes analyzed showed a high fold induction compared to their expression in the other estrous cycle phase. Genes upregulated in metestrus chosen for the validation were: CYP2C55 (*cytochrome P450, family 2, subfamily c, polypeptide 55*; fold induction over proestrus as measured in the microarray analysis: 1.94 with pVal=$5.7*10^{-4}$), ACSS2 (*Acetyl-coenzyme A synthetase*; fold induction over proestrus as measured in the microarray analysis: 2.08 with pVal=$6.8*10^{-3}$) and UPP2 (*uridine phosphorylase-2*; fold induction over proestrus as measured in the microarray analysis: 1.51 with pVal=$1.8*10^{-3}$); genes upregulated in proestrus chosen for the validation were: CYP2D9 (*cytochrome P450, family 2, subfamily d, polypeptide 9*; fold induction over metestrus as measured in the microarray analysis: 2.05 with pVal=$6.5*10^{-3}$) and CYP4A14 (*cytochrome P450, family 4, subfamily a, polypeptide 14* ; fold induction over metestrus as measured in the microarray analysis: 1.83 with pVal=$5.2*10^{-3}$).

The analysis with qPCR confirmed the data obtained from microarray intensities (Fig. 6).

Classification of genes differentially expressed based upon their biological function

The genes identified as differentially expressed in the two phases were then analyzed to see whether they fall into specific groups of biological functions, and therefore to see whether they share common functionality features.

To do this, the list of genes was used as template for the GO bionformatic analysis. GO (as Gene Ontology) is a bioinformatic initiative aimed in standardizing all the available information and terminology about genes and product of genes across species and databases (Osborne et al 2007). The GO project has developed three structured controlled vocabularies (ontologies) that describe gene products in terms of their associated biological processes, cellular components and molecular functions in a species-independent manner. Therefore, with the help of specific software, is possible

to interrogate a gene list to search for the most represented ontologies. For this analysis we used DAVID (Database for Annotation, Visualization and Integrated Discovery), a bioinformatic tool able to provide functional interpretation of large lists of genes derived from genomic studies (Huang da et al 2009). The upregulated in metestrus gene group and the upregulated in proestrus gene group were analyzed by DAVID algorithms and we obtained information about the enriched biological functions.

METESTRUS UPREGULATED GENES:

Gene Ontology Category	pValue	Most enriched genes
Lipid, fatty acid and cholesterol metabolism	5.10E-06	sterile alpha motif domain containing 8
		ATP citrate lyase
		cytochrome p450, family 2, subfamily r, polypeptide 1
		sterol-c5-desaturase (fungal erg3, delta-5-desaturase) homolog
		cytochrome b5 type b
		cytochrome p450, family 4, subfamily f, polypeptide 16
		mevalonate (diphospho) decarboxylase
		fatty acid synthase
		acetyl-coa synthetase
		farnesyl diphosphate synthetase
		phosphomevalonate kinase
		riken cdna 4632419c16 gene
		7-dehydrocholesterol reductase
		cytochrome p450, family 4, subfamily f, polypeptide 14
		cytochrome p450, family 2, subfamily c, polypeptide 55
		cdp-diacylglycerol synthase (phosphatidate cytidylyltransferase) 2
		acyl-coa synthetase long-chain family member 4
		elovl family member 6, elongation of long chain fatty acids
Detoxification	1.20E-04	ankyrin 3, epithelial
		glutathione s-transferase, mu 3
		7-dehydrocholesterol reductase
		glutathione s-transferase, alpha 2 (yc2)
		glutathione s-transferase, alpha 1 (ya)
		carboxylesterase 6
		estrogen receptor 1 (alpha)
		karyopherin (importin) beta 1
		glutathione s-transferase, mu 6
		glutathione s-transferase, mu 1
Steroid metabolism	1.30E-03	riken cdna 4632419c16 gene
		phosphomevalonate kinase
		7-dehydrocholesterol reductase
		riken cdna 1110020l19 gene
		cytochrome p450, family 4, subfamily f, polypeptide 14

		cysteine conjugate-beta lyase 1
		sterol-c5-desaturase (fungal erg3, delta-5-desaturase) homolog
		mevalonate (diphospho) decarboxylase
		lanosterol synthase

Table 1: In the table are shown the most enriched Gene Onthology (GO) categories for the genes upregulated during the metestrus phase, relative to their Biological Function.

PROESTRUS UPREGULATED GENES:

Gene Ontology Category	pValue	Most enriched genes
mRNA transcription regulation	4.80E-02	homeo box, msh-like 3
		transforming, acidic coiled-coil containing protein 1
		phosphatidylinositol-specific phospholipase c, x domain containing 3
		jumonji domain containing 3
		solute carrier family 25 (mitochondrial carnitine/acylcarnitine translocase), member 20
		sorting nexin family member 30
		zinc finger protein 410
Cell structure	8.00E-02	phosphatidylinositol-specific phospholipase c, x domain containing 3
		solute carrier family 25 (mitochondrial carnitine/acylcarnitine translocase), member 20
		sorting nexin family member 30
		trafficking protein, kinesin binding 1

Table 2: In the table are shown the most enriched Gene Onthology (GO) categories for the genes upregulated during the proestrus phase, relative to their Biological Function.

DAVID analysis indicates that during the metestrus phase, therefore in the phase where ovarian hormone levels are low in the bloodstream, there is a significant increase in the expression of genes connected with the regulation of fatty acid and cholesterol metabolism, and secondarily in the expression of hepatic genes involved in the detoxification and in the steroid metabolism. Notably, some of the genes identified as involved in fatty acids and cholesterol metabolism catalyze important steps of the same biosynthetic pathways, suggesting an additive estrogenic control in these important metabolic processes (Fig. 8). This group of genes, on the other hand, is not upregulated when we measure a peak of circulating estradiol; in this phase, the

proestrus phase, can be observed a rise in the genes whose biological function is linked with the mRNA transcription regulation in the cell nucleus.

This analysis, therefore, suggests that the oscillation of estrogenic levels during the four estrous cycle phase may impact the hepatic metabolic profile affecting gene expression.

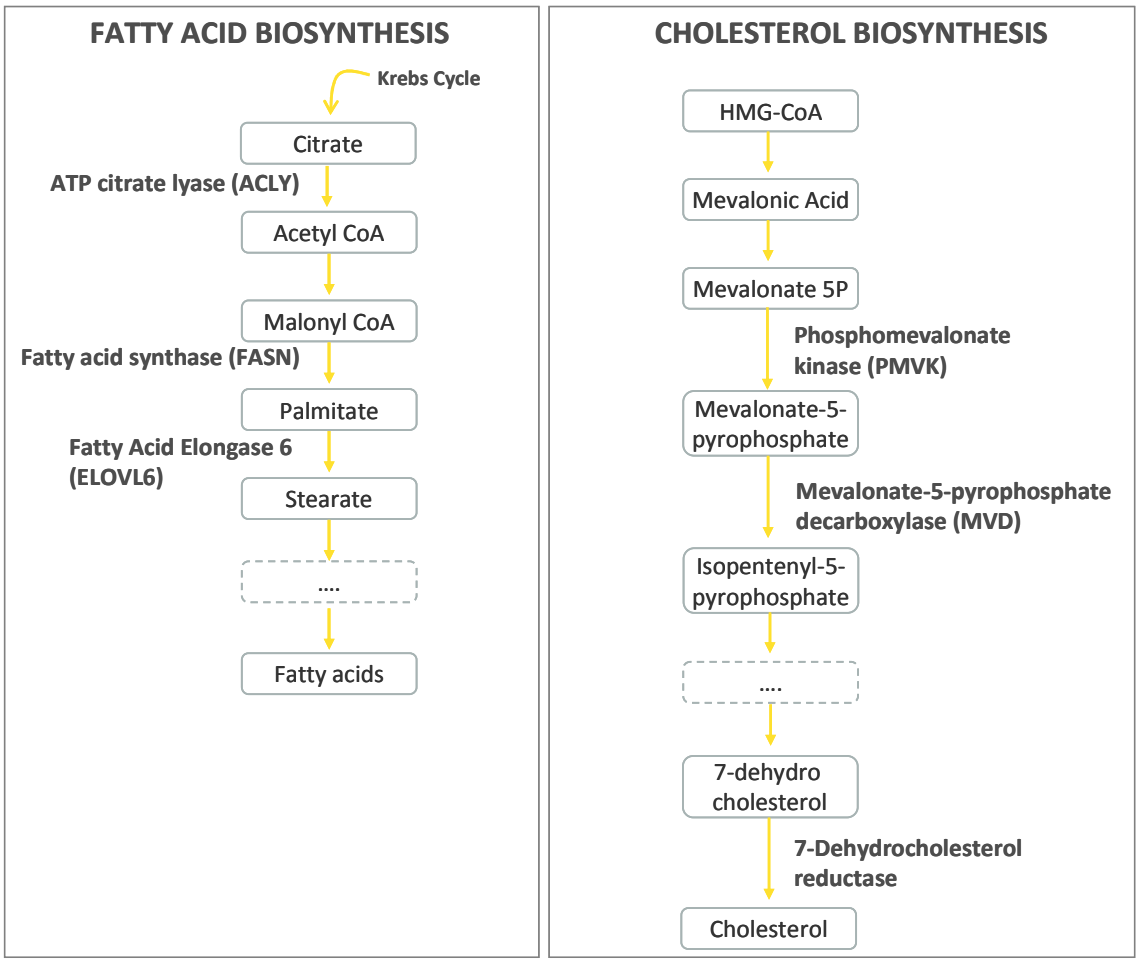


Figure 8 - Pathways of fatty acid and cholesterol biosynthesis. The genes identified as upregulated in metestrus and involved in lipid metabolism are indicated in the figure. Three of them (ACLY, FASN and ELOVL6) play an important role for the conversion of citrate derived from the Krebs cycle to de novo fatty acids; other three enzymes (PMVK, MVD and DHCR7) catalyze necessary steps of hepatic cholesterol biosynthesis.

ChIP-on-chip

After analyzing the genes differentially expressed, we decided to analyze the sites of Estrogen Receptor binding in the two conditions (proestrus and metestrus) to see whether subtle modifications in the estrogenic levels during the estrous cycle were sufficient to establish a positional shift in the localization of the receptor.

Optimization of the experimental parameters

ChIP-on-chip experiments are based on a complex series of steps, whose conditions need to be widely optimized. This is necessary to set the right parameters allowing for the best starting genomic material for the tiling array hybridization.

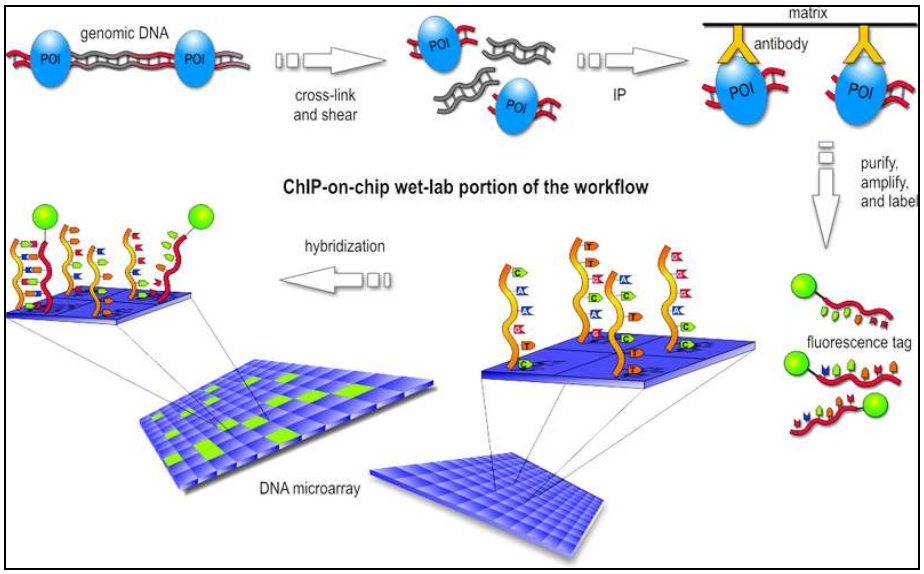


Figure 9 - Workflow of a ChIP-on-chip experiment: crosslinking of DNA and proteins (including the protein of interest, POI) with formaldehyde solution is followed by shearing through sonication and immunoprecipitation using specific antibodies directed against the protein of interest. DNA sequences bound by the protein are purified, amplified and labeled with a fluorescent tag, to be detected in the microarray analysis after hybridization (Wikipedia - Author: Thomas Hentrich).

Evaluation of the chromatin immunoprecipitation efficiency

Chromatin immunoprecipitation efficiency is generally measured as IP:input ratio for a given genomic region; this is defined as the amount of PCR product in the

immunoprecipitated (IP) sample divided by the amount of PCR product in the input sample, that is the original sample before the immunoprecipitation. This allow the quantitative measurement of the efficiency of the immunoprecipitation technique: the higher is the enrichment of the region of interest in the IP sample compared to the input sample, the higher is the efficiency.

Usually, genomic regions of interest are analyzed in the same reaction mixture with a constant "reference region" that serves as an internal normalization control; this is indicated as "intergenic region" and is a particular region known not to be bound by the transcription factor to be immunoprecipitated (estrogen receptor in this case).

Optimization of the crosslinking timing

Crosslinking with formaldehyde is the first step of the procedure, and is necessary to create a heat-reversible bond between the DNA and the proteins interacting with the double helix. The harvested tissue, liver in this case, is cut into small pieces to allow the permeation at the crosslinking solution to every cell and is then soaked in 1% formaldehyde solution at 37°C. To determine what the best time range for crosslinking is, a single animal liver was cut into small pieces, and tested in parallel: liver tissue was placed at 37°C in crosslinking solution for 5', 10', 15' and 20 minutes. After removing the tubes from the heat room, crosslinking was stopped and we continued in the same procedure for the 4 samples. The quantification of the immunoprecipitated DNA samples and the IP:input ratio confirmed that 15 minutes was the most efficient timing for crosslinking.

Optimization of the sonication conditions

Sonication is a necessary step to shear the genomic strands into fragments of 100-500 base pairs. Best sonication conditions depend on a number of parameters, which includes: the type and model of the sonicator machine, the machine settings, the length of sonication, the dilution of DNA into lysis buffer and the volume of the sonication substrate. Different conditions have been tested in parallel to find the best combination

to achieve the highest efficiency and the best fragment length. Sonication quality and efficiency was checked by running 100-500 ng of purified DNA sample on an agarose gel.

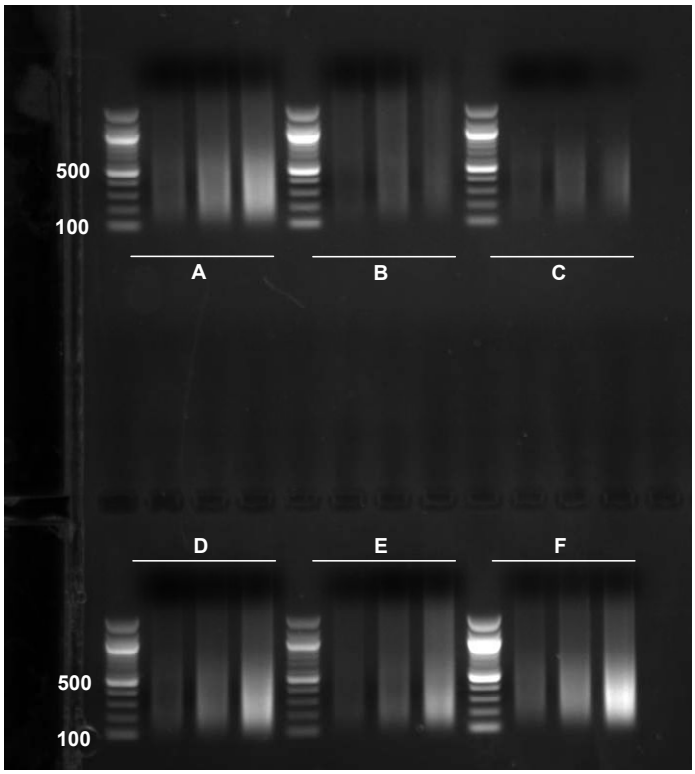


Figure 10 - Agarose gel electrophoresis (2% agarose) with 100bp ladder. Each group with three lanes (from A to F) represent three different concentration of samples treated with different sonication conditions. Condition F, 20% amplitude, 10 times 10 seconds each, 30 or more seconds pause on ice, allowed to obtain the best fragments and was therefore used in the experiment.

Eventually the sonication step gave the best results using the tip sonicator with the following conditions: 20% amplitude, 10 times 10 seconds each, 30 or more seconds pause on ice.

Optimization of the antibodies conditions

Finally we checked the efficiency of the chromatin immunoprecipitation with different antibody concentrations and mix. Antibody must be highly specific and allow for an efficient precipitation of the protein of interest, with very limited precipitation of non-specific targets. In literature and in previous experiments carried out in our laboratory,

the most widely used antibodies for estrogen receptor alpha immunoprecipitation are MC-20 (sc-542, Santa Cruz Biotechnology) and Ab-10 (Lab Vision, Thermo Scientific). Therefore I planned different experiments to check the immunoprecipitation enrichment obtained with several combinations.

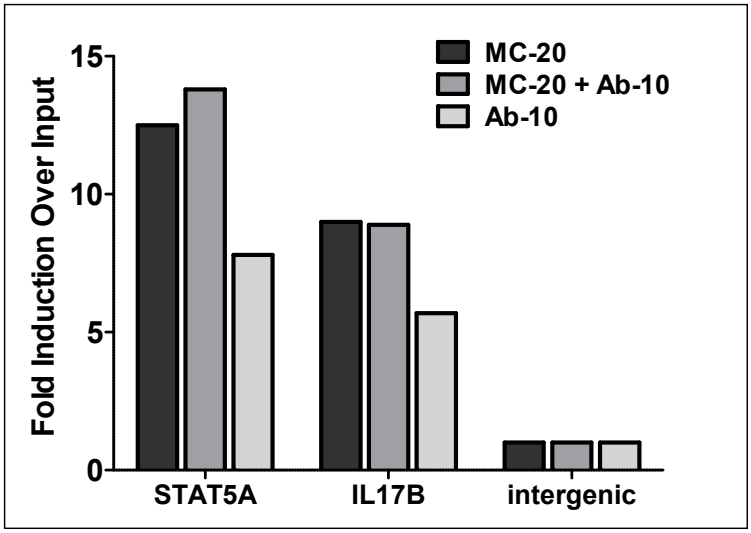


Figure 11 - Graph reporting enrichment of ER-bound fragments compared to non ER-bound fragments (intergenic), to test the antibodies immunoprecipitation efficiency.

Experiments proved that the use of Ab-10 in combination with MC-20 did not improve the selective precipitation performance; the highest enrichment tested on selected estrogen receptor-bound sequences was obtained with 2 μg antibody MC-20 diluted with the samples in about 2 ml dilution buffer.

ChIP-on-chip bioinformatic analysis reveals two sets of Estrogen Receptor-bound sequences

Bioinformatic analysis of the intensities detected by the tiling arrays image acquisition was performed in collaboration with Shirley Liu's Lab (Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Harvard School of Public Health, Boston, MA, USA) using MAT algorithms.

This analysis revealed 553 high-affinity sequences bound by ERα during the metestrus phase, and 444 sequences bound by ERα during the proestrus phase. The identified localization of ERα are widely distributed over the mice chromosomes (Fig. 11)

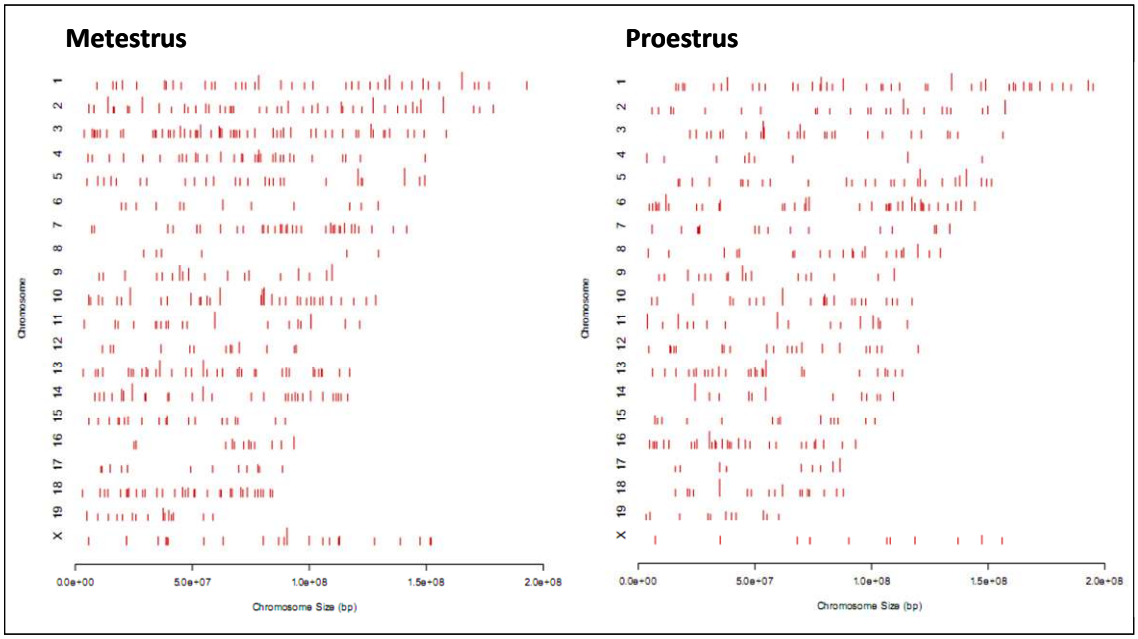


Figure 12 - Distribution over the mice chromosomes of the identified Estrogen Receptor alpha binding sites.

The two phases share only the 15% of the binding sites identified (Fig. 12). This suggests that the subtle differences in estradiol concentration in the bloodstream between the two phases might be sufficient to determine a shift in the genomic localization of the activated estrogen receptors.

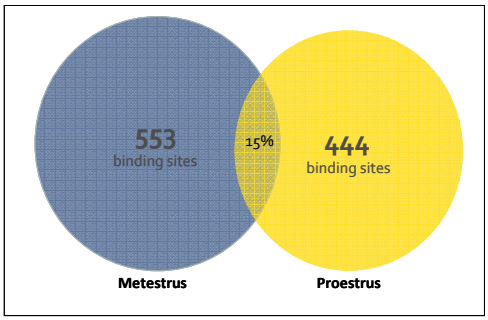


Figure 13 - Venn diagram showing the common identified Estrogen Receptor binding sites in the two phases.

Selected sequences identified can be validated with qPCR

To confirm that the sequences identified with the analysis of the tiling arrays were indeed enriched if compared to input sample, we selected different fragments to be validated with the more sensitive Real Time PCR.

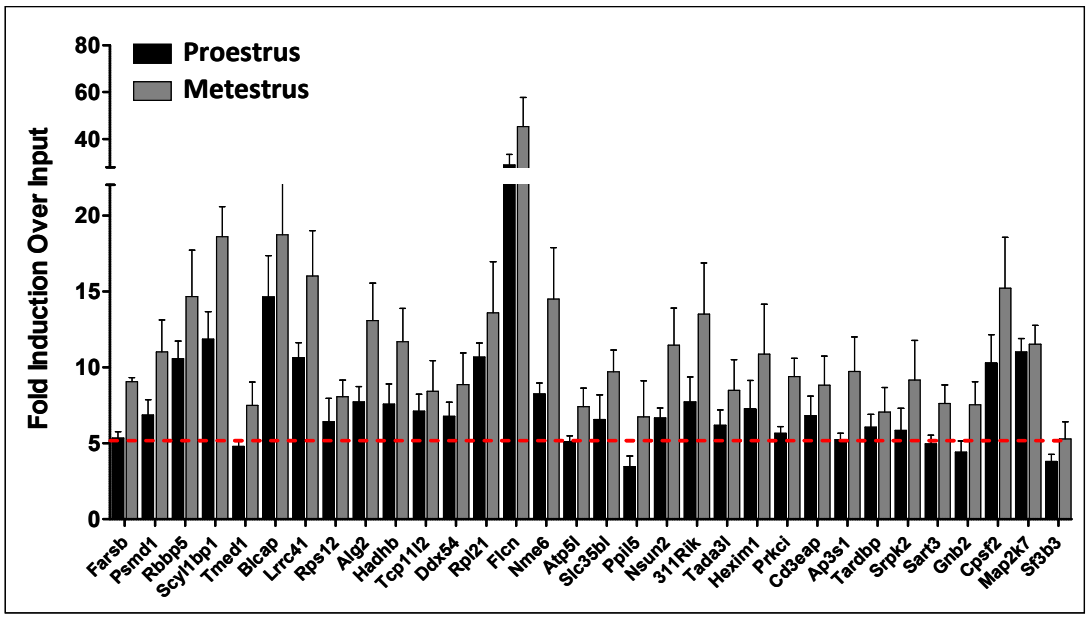


Figure 14 - Validation of the ChIP-chip analysis: identified ER-bound sequences have been analyzed with quantitative PCR to assess the enrichment of those particular sequences after immunoprecipitation compared to input (before immunoprecipitation). The fragments are considered adequately enriched when their induction over input is greater than 5.

The majority of the selected sequences were validated, considering validated when expressing a fold induction over input greater than five fold.

Localization of the Estrogen Receptor-bound sequences

After identifying the sequences bound by Estrogen Receptor in the two conditions, and after defining that their localization over the chromosomes changes with the level of

estradiol in the bloodstream, we further analyzed the binding sites to determine their localization relative to known genes.

Transcription factors can indeed bind in different genomic domains, based upon their function. These binding sequences can be close to the gene TSS (transcription Start Site) or at many bases or kilobases away.

Binding sites can localize at promoter (from the TSS to 3000 bp upstream), at distal intergenic (> 3000 bp upstream), in the gene sequence, both at intronic or exonic sequences, or at 5' or 3' UTR, and downstream.

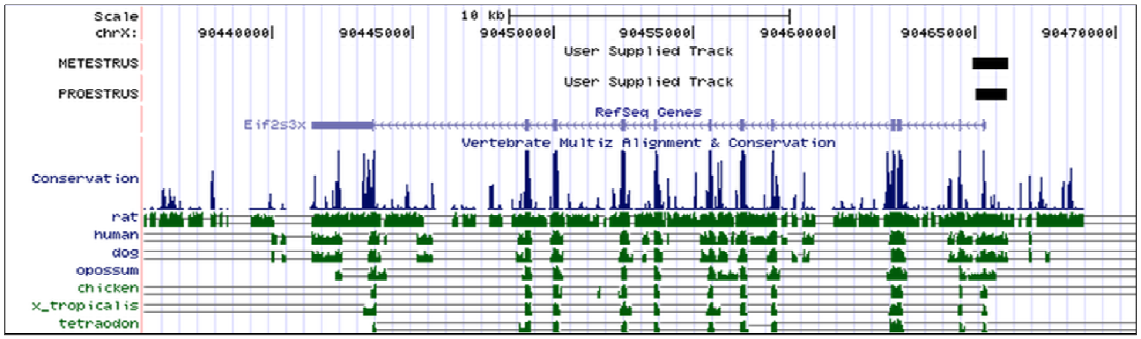


Figure 15 - Screenshot from the UCSC genome browser (http://genome.ucsc.edu). This browser is a graphical viewer of genome sequence and conservation data from a variety of vertebrate and invertebrate species; it allows to load custom tracks (in this case Proestrus and Metestrus ER-bound sequences) and to visualize them on the genome. Here is reported an exemplificative screenshot, showing the Eif2s3x gene promoter localization of two indentified ER-bound sequences.

The analysis of the binding sites identified in proestrus and metestrus indicates that the majority of the binding sites localizes in distal intergenic regions, in agreement with the recent literature; a big slice (27-28%) of the sequences is found in the intergenic regions, while 18% of proestrus sequences and 12% of metestrus sequences are localized in the promoter of active and known genes.

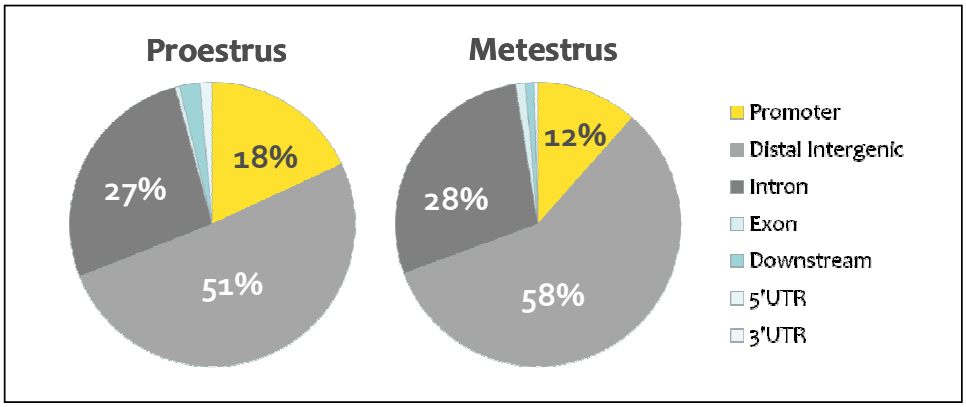


Figure 16 - Pie chart expressing the localization of the estrogen receptor binding sequences identified.

The localization at the promoter is less if compared with the distal intergenic regions or with the intronic region; however appear significantly higher if considered the localization of estrogen receptor in the genome of MCF-7 cells estradiol treated (binding sites at the promoter region: 4%) or in liver of ovariectomized females injected with 100 μg/Kg estradiol (binding sites at the promoter region: 8%).

The binding at the promoter regions does not appear as a casual event: indeed the fraction of ERs localized in the promoter region in proestrus and metestrus samples is significantly higher than random sequences, and appears to be also increased compared with the fraction of sequences bound by ER identified in the ovariectomized estradiol-treated samples (Gao et al 2008) and in MCF-7 cells (Carroll et al 2006).

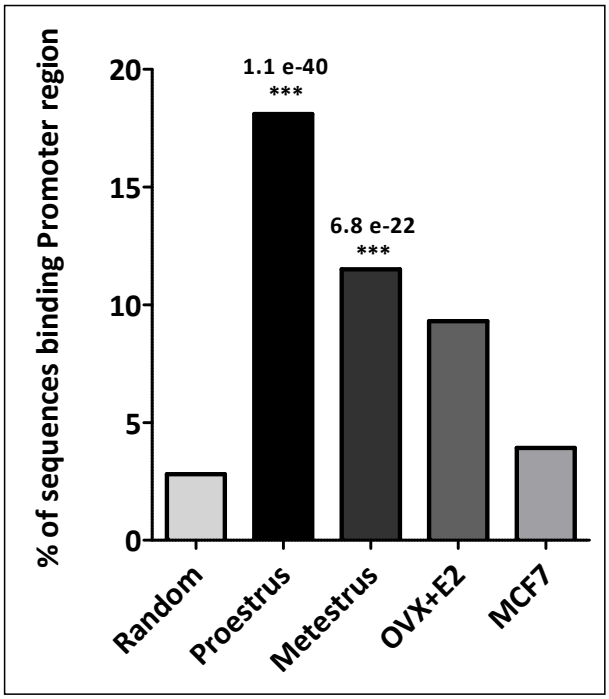


Figure 17 - Graph showing the percentage of ER-bound sequences that localize in the promoter region in different conditions (Proestrus phase, Metestrus phase, ovariectomized animals treated with 100 μg/kg estradiol after 2 hours, MCF-7 cells treated with 100 nM estradiol after 45 minutes).

Genes nearby Estrogen Receptor-bound sequences have distinctive biological functions in the two phases

Gene expression is generally regulated thanks to the specific transcription factor binding to the promoter regions of DNA adjacent to the genes that they regulate and recruiting the pre-initiation complex for transcription start. Therefore, after the identification of the ER-bound sequences, we wanted to check whether could be observed a correlation between the genomic localization of the activated estrogen receptors, and the genes that appear to be differentially expressed in the two phases.

We listed every gene that appears to be localized in the proximity of the receptor bound sequences, considering as proximity a 20 kilobases distance each side. Once identified the genes confined in the 20 kb surroundings of the ER-bound sequences, we examined if these genes matched with the regulated ones. The analysis failed to identify a significant correlation between the sequences identified with the ChIP-on-

chip analysis and the genes differentially expressed discovered with the microarray profile.

However, when we analyzed the Gene Ontology biological functions of the genes in the proximity of ER-bound sequences in the two phases, we noticed that matched with the biological functions of the previously identified regulated genes: particularly, known genes that fall in the 20 kb surroundings of ER-bound sequences in proestrus are involved in cellular processes and specifically in gene expression regulation (pVal= $8.50e^{-05}$), while known genes that fall in the 20 kb surroundings of ER-bound sequences in metestrus are associated with the metabolic processes and specifically in lipid metabolic processes and hormone level regulation (pVal= $1.40e^{-03}$).

Table 3 - Gene ontology categories of genes that fall in the 20 kb surroundings of ER-bound sequences in Metestrus.

Gene Ontology Category	pValue	Number of enriched genes
cellular process	1.20E-03	178
metabolic process	1.30E-03	142
regulation of hormone levels	1.40E-03	9
lipid metabolic process	1.40E-03	24

Table 4 - Gene ontology categories of genes that fall in the 20 kb surroundings of ER-bound sequences in Proestrus.

Gene Ontology Category	pValue	Number of enriched genes
cellular metabolic process	1.10E-06	138
metabolic process	6.50E-06	155
gene expression	8.50E-05	69
RNA metabolic process	4.70E-03	22
transcription	5.70E-02	40

This suggests that, even if we failed to reveal a precise correlation with regulated genes and transcription factor binding, the fluctuation of the hormone levels during the estrous cycle cause a profound metabolic shift, reflected in the expression of particular categories of genes and in the estrogen receptor activated localization on the genome.

Evaluation of the levels of the differentially expressed genes in physiological conditions

After the analysis described above, we wanted to investigate whether the genes connected with the lipid and cholesterol metabolism, upregulated in liver during the metestrus phase, and the genes associated with the transcription regulation, upregulated in liver during the proestrus phase, would change their levels of transcription in other physiological conditions of the female mouse life.

Expression of the differentially expressed genes oscillates during the estrous cycle phases

First, we chose a representative set of genes for each expression group, to be tested in the experiments described below. For the group of genes upregulated during the metestrus phase, were chosen three genes important for the triglycerides metabolism (FASN, *Fatty Acid Synthase*; ACLY, *ATP Citrate Lyase*; ELOVL6, Elongation *Of Long Chain Fatty Acids, Family Member 6*) and three genes necessary for the cholesterol biosynthesis (MVD, *Mevalonate Diphospho Decarboxylase*; PMVK, *Phosphomevalonate Kinase*; DHCR7, *7-Dehydrocholesterol Reductase*); on the other hand, for the group of genes upregulated during the proestrus phase were selected: KDM6B (*Lysine (K)-Specific Demethylase 6B*), CYP4A14 (*Cytochrome P450, Family 4, Subfamily A, Polypeptide 14*), ZNF410 (*Zinc Finger Protein 410*) and TACC1 (*Transforming, Acidic Coiled-Coil Containing Protein 1*).

Since in the microarray analysis we tested the differences in expression between two discrete moments (proestrus, high estrogens; metestrus, low estrogens), the first experiment with quantitative PCR technique focused in the definition of the real pattern of expression of the differentially expressed genes during the four phases of the estrous cycle.

Data in Fig. 17 and 18 shows that the transcript levels of the identified differentially expressed genes indicate a clear and regular oscillatory pattern of expression following the variation of circulating estrogen levels in intact cycling females.

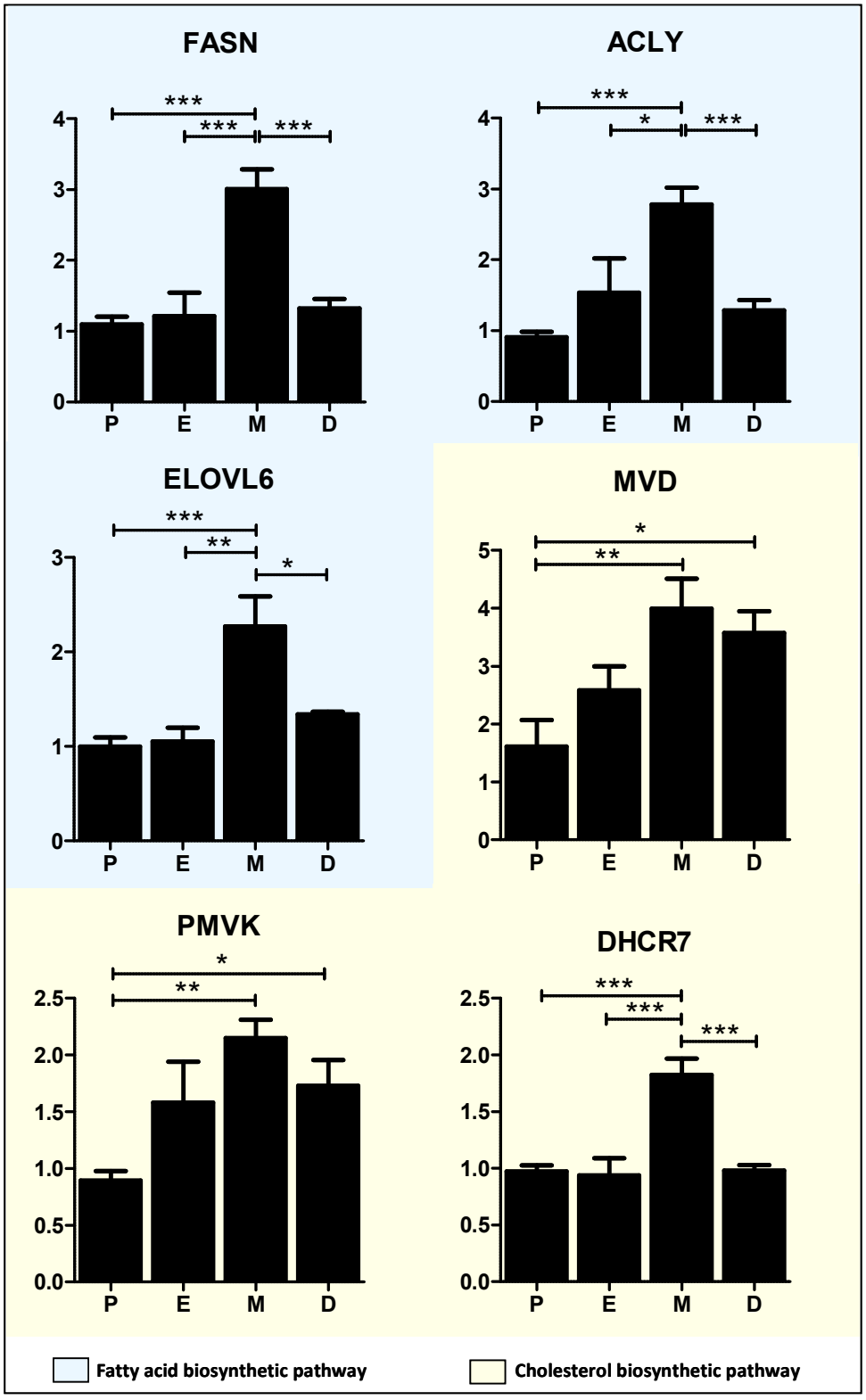


Figure 18 - Graphs reporting the mRNA level of the genes upregulated in metestrus during the four estrous cycle phases. In blue background are the genes involved in fatty acids biosynthetic pathways, in yellow background the genes associated with cholesterol biosynthetic pathways.

Each group consists of three animals. Statistical analysis was performed with one way ANOVA followed by Bonferroni post-hoc test; *=0,05>pVal>0.01; **=0,01>pVal>0,001; ***=pVal<0,001.

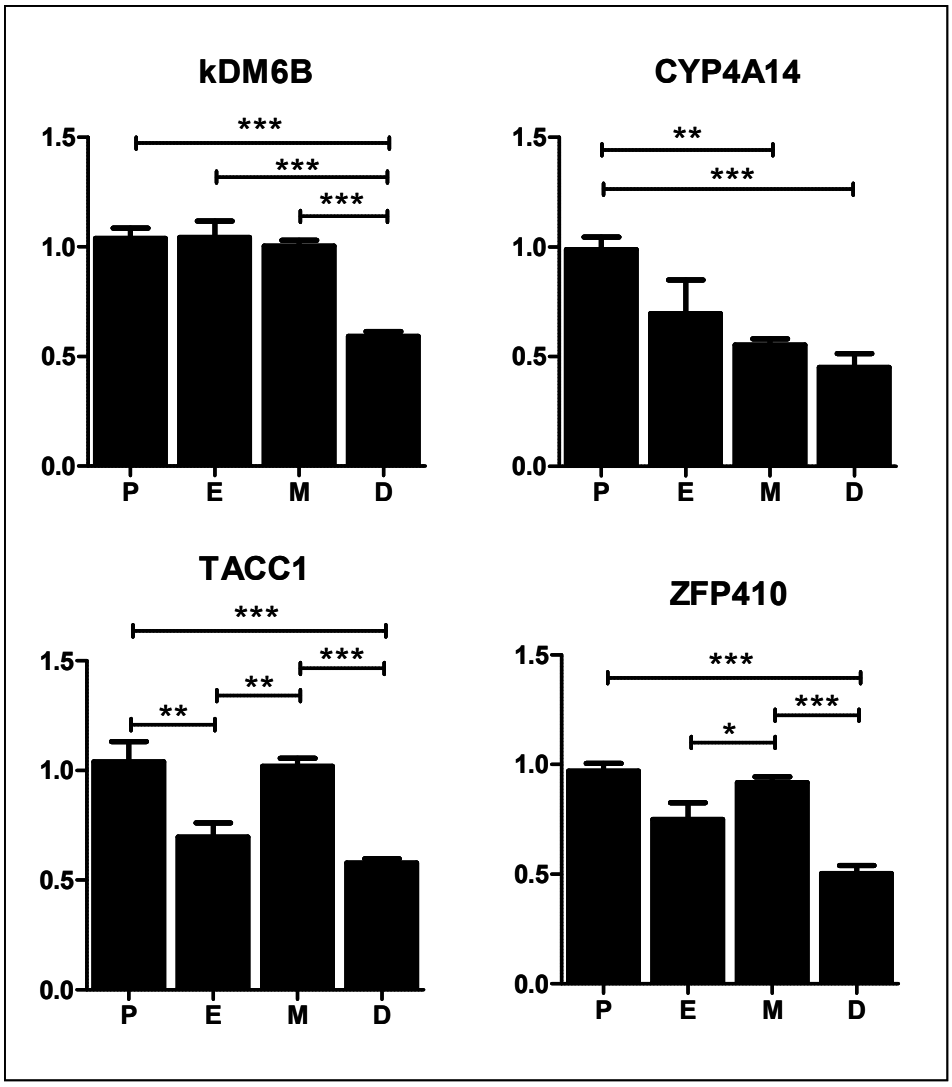


Figure 19 - Graphs reporting the mRNA level of the genes upregulated in proestrus during the four estrous cycle phases. Each group consists of three animals. Statistical analysis was performed with one way ANOVA followed by Bonferroni post-hoc test; *=0,05>pVal>0.01; **=0,01>pVal>0,001; ***=pVal<0,001.

Upregulated genes during metestrus are negatively regulated by estradiol treatment

Once established the pattern of expression of the selected genes, we decided to check whether the same genes appear to be directly regulated by the estrogenic action. In order to test this hypothesis, we ovariectomized female adult mice, and, two weeks after the ovariectomy, we injected them with vehicle (corn oil) or 50 μg/kg 17-β-estradiol, and we analyzed the mRNA level of the reference genes 6 and 24 hours after treatment.

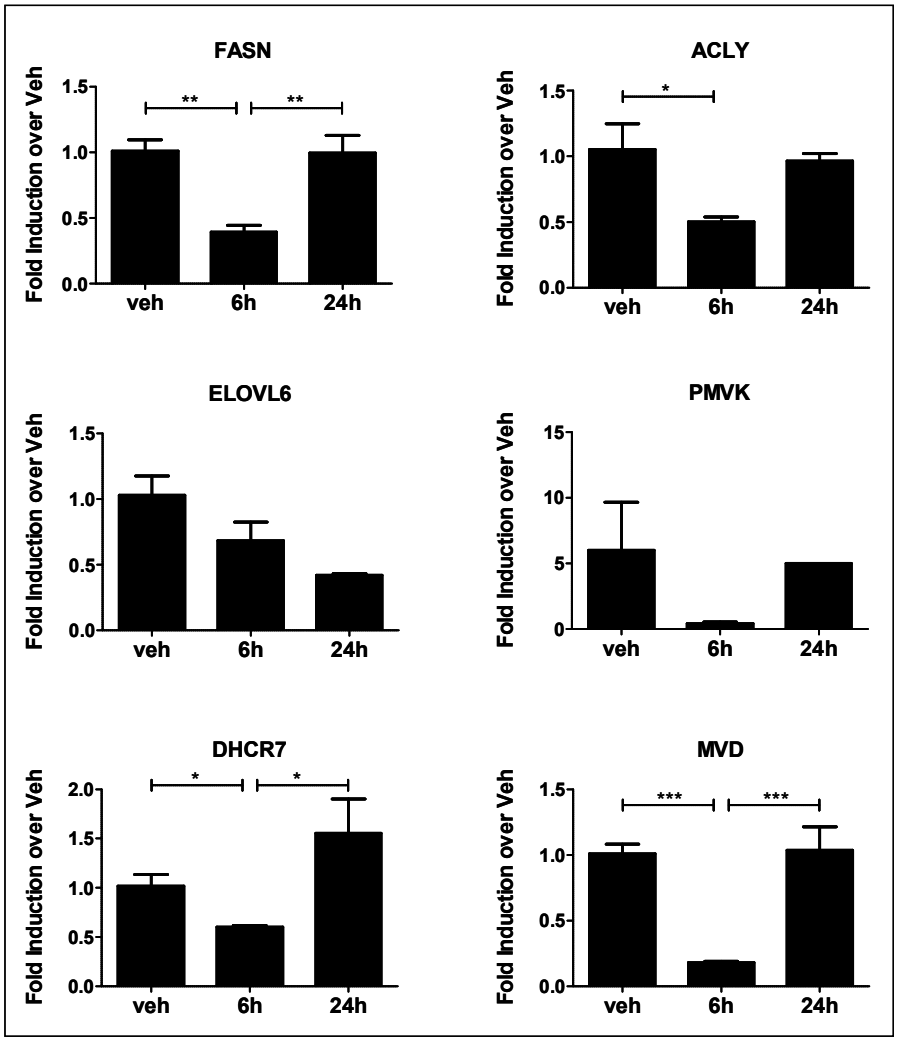


Figure 20 - Graphs reporting the mRNA level of the genes upregulated in metestrus after estradiol treatment. 50 μg/Kg 17-β-estradiol and vehicle (corn oil) were injected subcutaneously, and livers were harvested 6 and 24 hours post-treatment. Vehicles livers harvested at 6 and 24 hours did not show any variation (not shown). Each group consists of three animals. Statistical analysis was performed with one way ANOVA followed by Bonferroni post-hoc test; *=0,05>pVal>0.01; **=0,01>pVal>0,001; ***=pVal<0,001.

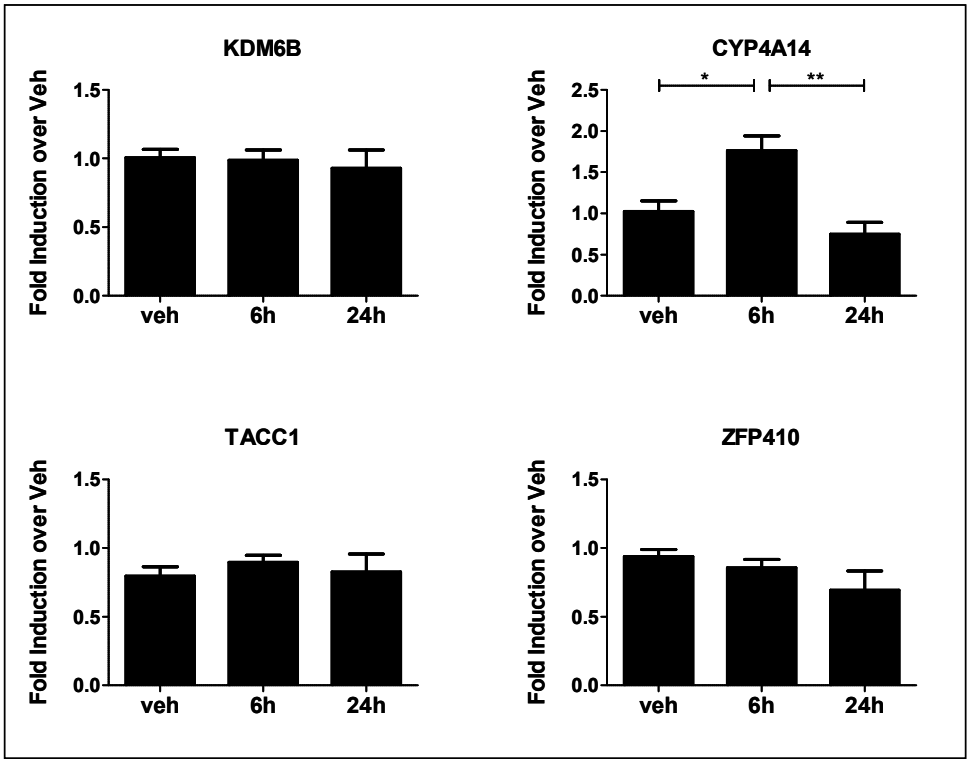


Figure 21 - Graphs reporting the mRNA level of the genes upregulated in proestrus after estradiol treatment. 50 μg/Kg 17-β-estradiol and vehicle (corn oil) were injected subcutaneously, and livers were harvested 6 and 24 hours post-treatment. Vehicles livers harvested at 6 and 24 hours did not show any variation (not shown). Each group consists of three animals. Statistical analysis was performed with one way ANOVA followed by Bonferroni post-hoc test; *=0,05>pVal>0.01; **=0,01>pVal>0,001; ***=pVal<0,001.

In agreement with the pattern observed during the estrous cycle, the genes whose level appears to be lower in proestrus (high estrogens) and higher in metestrus (low estrogens) are indeed negatively regulated after hormone injection in ovariectomized animals. On the contrary, the genes upregulated in proestrus, with CYP4A14 exception, does not show a clear estrogen-dependent expression.

Expression of differentially expressed genes in other physiological conditions

Ultimately our goal was to investigate on the level of expression of the genes, which appear to be regulated during the reproductive period in intact female mice, in other physiological conditions of the female life cycle.

Namely, we checked their mRNA level in: prepuber mice, at 20 days old, characterized by low estrogenic levels; pregnant mice, at 17th day post conception, with vey high estrogenic levels in the bloodstream; in old mice, at 22 months old, when circulating hormones drop again.

In Fig. 22 we can observe the physiological levels of the genes involved in lipid (fatty acids and cholesterol) biosynthesis, more expressed during the metestrous phase. The transcripts of these genes appear to be significantly downregulated in the liver of pregnant mice at 17th day post-conception, where the estradiol levels in the bloodstream are indeed considerably high. However, in prepuber mice and in 22 months old mice, the levels are higher, although only significantly higher in old mice compared to proestrus.

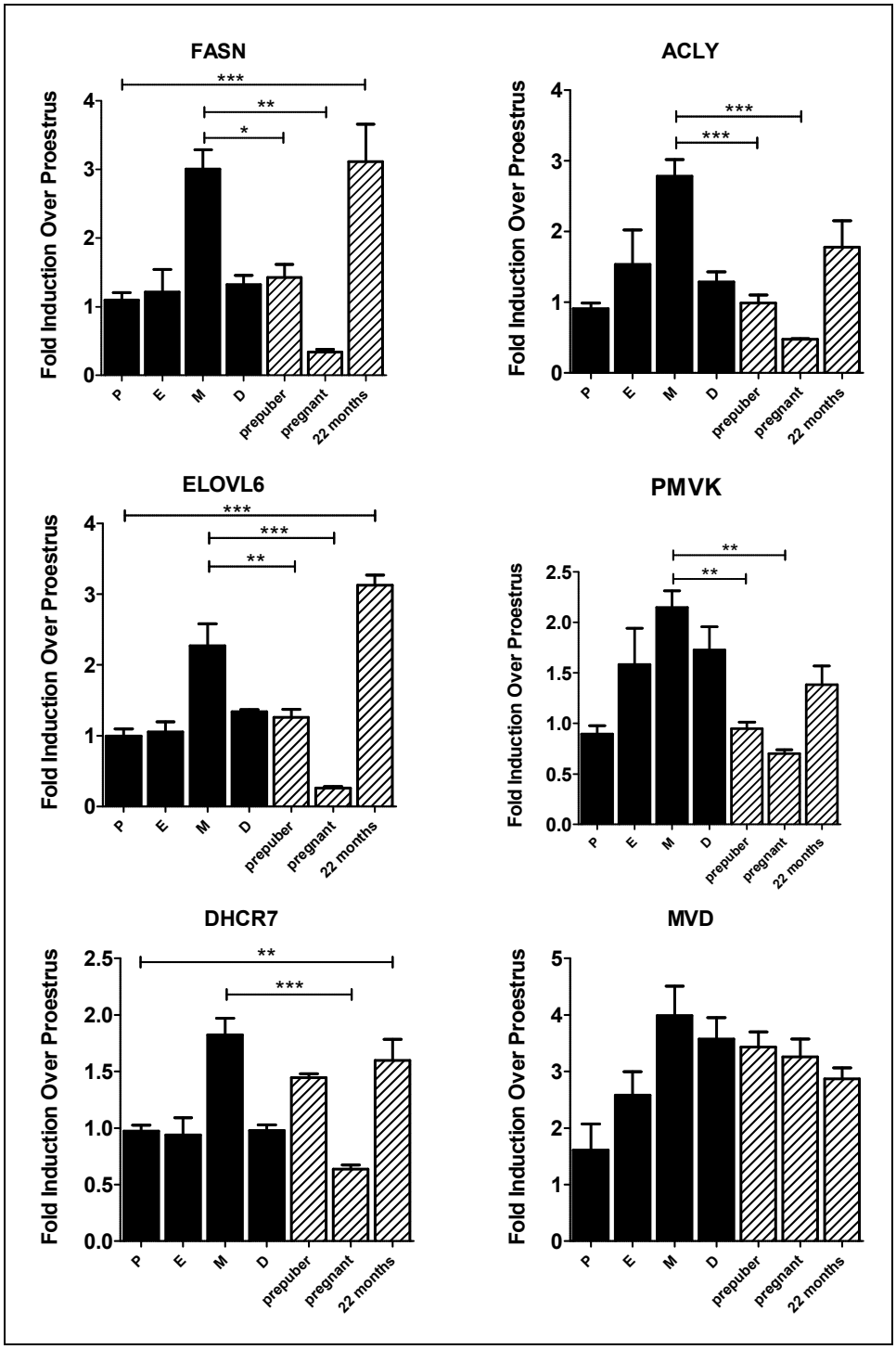


Figure 22 - Graphs reporting the mRNA level of the genes upregulated in metestrus during the four estrous cycle phases, and in prepuber, pregnant and old female mice. Each group consists of three animals. Statistical analysis was performed with one way ANOVA followed by Bonferroni post-hoc test; *=0,05>pVal>0.01; **=0,01>pVal>0,001; ***=pVal<0,001.

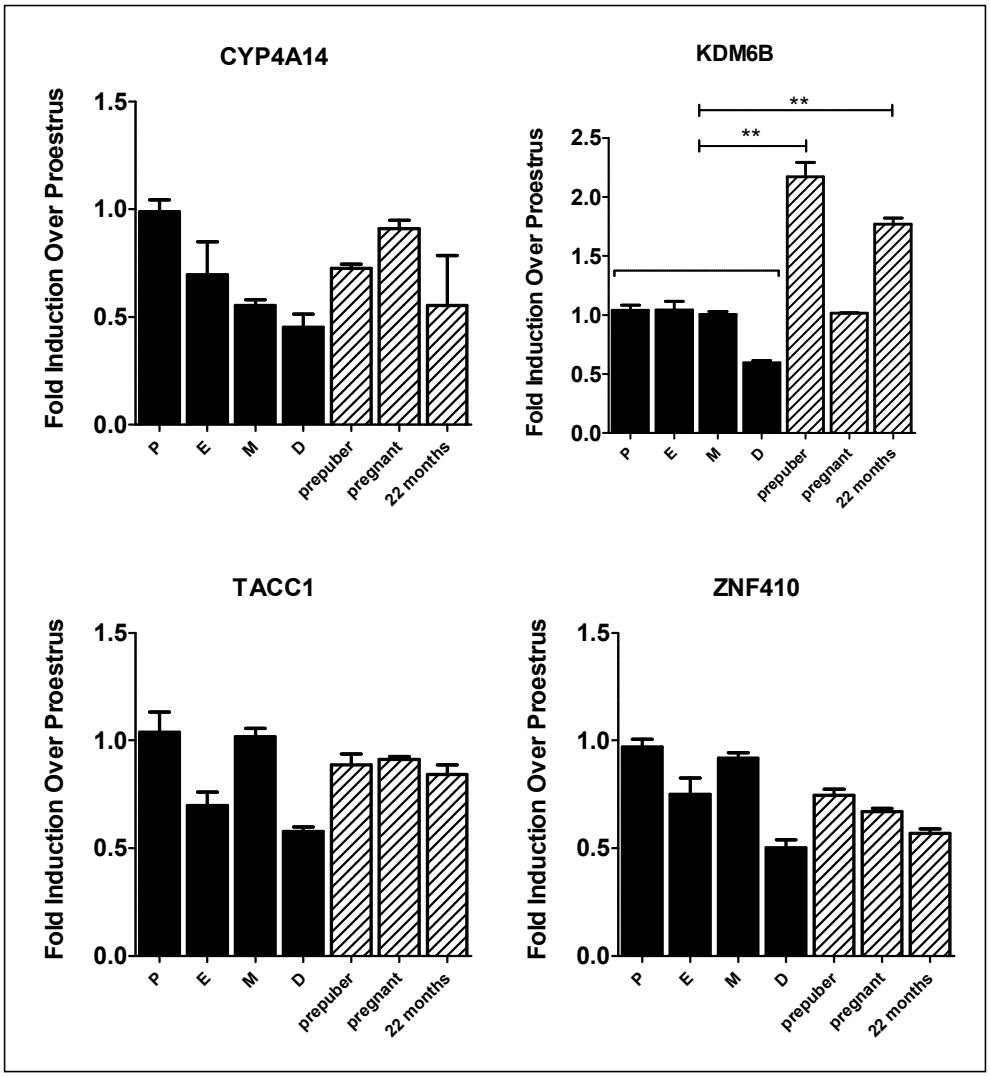


Figure 23 - Graphs reporting the mRNA level of the genes upregulated in metestrus during the four estrous cycle phases, and in prepuber, pregnant and old female mice. Each group consists of three animals. Statistical analysis was performed with one way ANOVA followed by Bonferroni post-hoc test; *=0,05>pVal>0.01; **=0,01>pVal>0,001; ***=pVal<0,001.

8. Discussion

Estrogens, the hormones connected with the control of the reproductive activities, appear to play a key role in the regulation of lipidic and glucidic metabolism in liver tissue, as indicated in studies on diabetic ob/ob mouse models (Gao et al 2006), or as suggested by the deregulation of the lipidic profile during menopause. According to the gender studies carried out over the past decade, it is well established that females appear to be more protected from hypercholesterolemia-related diseases, such as cardiovascular diseases (CVD), in the pre-menopausal period (Maxwell 1998). CVD risk increases after the menopause, and in many women features of the metabolic syndrome (abdominal adiposity, insulin resistance, and dyslipidemia) emerge with estrogen deficiency. Therefore, female protection may be principally mediated by the hypolipidemic properties of estrogens (Farhat et al 1996); indeed, high levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triglycerides (TG), and TC/high-density lipoprotein cholesterol (HDL-C) were associated with menopause (Fukami et al 1995, Graff-Iversen et al 2008). Not surprisingly, women taking hormone replacement therapy show a reduction in LDL cholesterol levels (Dallongeville et al 1995, Skafar et al 1997).

However, the mechanisms underlying this estrogenic regulation on hepatic cholesterol and lipids are still unclear. Some studies tried to focus on the hormonal action on the cholesterol biosynthesis rate-limiting enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR), but available data about estrogen effects on HMG-CoAR regulation are yet controversial (Carlson et al 1980, Marino et al 2001). For this reason, we focused our investigations on the adult and cycling female mouse, to highlight what are the physiological influences of the ovarian hormones in the hepatic tissue of healthy organisms. Microarray analysis and chromatin immunoprecipitation on tiling arrays (ChIP-chip) are therefore the methodologies exploited in this study, to see whether the subtle oscillations of estrogenic blood levels during the estrous cycle may

impact first on the expression pattern in liver, and second on the localization of activated estrogen receptors. Data presented here show that during the reproductive cycle, the variations of estradiol levels have a strong effect in the transcription of specific hepatic genes. Notably, a significant group of genes regulated by estrogens are involved in lipid metabolic processes, particularly fatty acids and cholesterol biosynthesis. The expression pattern of this group of genes appears to be regularly cycling during the reproductive phases, with a reduced transcription at proestrus (peak of estradiol in the bloodstream) and an increased transcription at metestrus (low levels of estrogens). On the other hand, the highest expression during the proestrous phase is observed for genes linked with transcription regulation. A sharp differentiation in the hepatic metabolic profile appear thus to be induced by the changes in estradiol concentrations during the four-day cycle. The microarray expression findings are mirrored in the data obtained with the ChIP-chip experiments: ligand-activated estrogen receptors localize indeed in the 20 kilobases surrounding of genes involved in lipid metabolism at metestrus, while at proestrus they localize in the 20 kilobases surrounding of genes controlling transcription.

In this way, we were able to precisely characterize the steps in the lipid biosynthetic pathway which are regulated by estradiol levels in physiological conditions, and particularly we identified six genes upregulated in metestrus, three involved in cholesterol biosynthesis, and three involved in fatty acids biosynthesis, representative of the "healthy" status.

Therefore we can deduce that the ovarian hormones, secreted during the estrous cycle and regulating the reproductive functions, may also influence the hepatic pathways associated with the control of the energetic metabolism. This association can play a key role in the adaptation of the energetic needs with the reproductive status, and in adapting the female organism for a potential pregnancy. Indeed the higher expression of a *de novo* cholesterol biosynthetic enzymes during the last part of the estrous cycle, when hormone circulating levels are low, is in agreement with the gonadic demand for the steroid substrate: cholesterol is produced by liver in metestrus-diestrus, in order to

be available in the ovaries on time for the generation of the 17β-estradiol surge at proestrus. Again, the increased fatty acid biosynthetic pathway during metestrus may be a consequence of the animal feeding behaviour during estrous cycle; in fact it has been documented a change in the food intake in rodents during the estrous cycle progression: particularly, animals show a peak in food consumption during the estrous phase, and a lower consumption in metestrus and diestrus phases (Todd et al 2007). Since *de novo* fatty acid synthesis derives from a glucose excess from diet, which is metabolized to Acetyl-CoA in the Krebs cycle, it is expected that when the food consumption is reduced, also this metabolic pathway is inhibited. However, this pathway becomes of fundamental importance in other conditions, for example in the last third of gestation. Gestation can indeed divided, from the hormonal and metabolic point of view, in two stages: the first one, coinciding with the first two thirds, is characterized by a limited foetal growth and by a mother accumulation of fat stores; the second one, corresponding with the last third, is characterized by rapid foetal growth, and by an enhanced transfer of nutrients throughout the placenta (Lopez-Luna et al 1986, Villar et al 1992). The hormonal levels are low in the first stage and they steadily increase in the second stage, inducing a lipid catabolic condition: this reflects the fact that all the fat that has been stored in the first stage must be broken down, allowing glucose to be available for the growing foetus. Indeed, placenta permeability to lipids is quite limited, while glucose is the most important substrate crossing it thanks to GLUT transporters (Aldoretta & Hay 1995).

Prebuberty and menopause profiles show a sustained expression of genes involved in the lipid biosynthetic pathways; this is, as expected, clearly higher than the levels observed in the pregnant mice. However, in prepuber mice, the maximum expression is not as high as during metestrus, while is comparable to the transcript level measured in the proestrus phase. On the other hand, supporting the hypothesis of these gene involvement in the dyslipidemic profile observed in menopause, the expression level of lipid and cholesterol biosynthetic genes in menopause mice appears significantly higher than proestrus.

Therefore this study points out the possible mechanism that link the reproductive state with the control over the energetic metabolism, particularly liver lipidic metabolism. Once established the basal picture of expression, we are able to define if the normal and "healthy" pattern is altered in other conditions of the female life cycle. This is important to precisely identify pharmacological targets, to design a more focused intervention aimed to re-establish the appropriate energetic metabolism in the post-menopause.

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Ringraziamenti:

Una grazie alla Prof.ssa Maggi, per il supporto lavorativo ed extralavorativo che non è mai venuto meno, e per tutte le opportunità di crescita che mi sono state concesse.

Un grazie enorme a tutto il gruppo, ormai quasi tutto al femminile (saranno tutti questi estrogeni a creare una barriera impermeabile all'uomo??), che ha lavorato con me in questi anni

Grazie mille alle mie altre due compagne di Dottoranza: la Vale e la Guiseppa, che ancora forse mi sta maledendo per averle lasciato il posto; a Clara e a Sara, che si è aggiunta come colonna portante del mondo Maggi; alle due ex-compagne di merende: Cri ed Eli. C'eravate tutte all'inizio, ci siete ancora, siete state parte integrante della mia vita di questi anni e non sarebbe stato tutto così piacevole senza le risate, senza i pettegolezzi, senza il supporto e senza le confidenze che abbiamo condiviso.

Grazie mille a Giampa e Balaji, oramai lontani ma ancora ben radicati nei miei ricordi di questo laboratorio.

Grazie a tutti gli studenti che vanno e che vengono, e che rendono questo posto vivo: a Giulia, a Silvia, al buon vecchio Alberto e naturalmente all'ultimo vero uomo rimasto (anche se il soprannome non sostiene molto questa affermazione) Luca.

Grazie a tutto il piano alto, a Paolo ed Elisabetta, a Rossana che veglia sui nostri macelli e a Isabbè, compagna di mille avventure in casa e fuori casa, la nostra cara vecchia (?) Rotondo.

Grazie a tutto il gruppo dall'altra parte dell'oceano: a Myles, a Mathieu il mio spirito guida, a Jennifer e tutto il suo entusiasmo, a Cliff, ai suoi racconti fantastici e a tutto il gruppo di nerd bioinformatici, a Shannon e le sue certezze metodologiche, a Luz e a Marisa le mie donne latine, a Jin la tecnica più veloce del West, a Min, a Hanson, a Tom, a tutti quelli che mi hanno aiutato con una disponibilità che è difficile incontrare altrove.

Grazie alle tantissime figure che invece hanno reso speciali, fuori dal lab, i mesi lontani da casa, e un grazie col cuore a Martina ed Elena, Yuliya, Erin, Eli, Tony.

Un grazie speciale, infine, a chi mi è vicino sempre: a mamuth e a papi, a Tia, a Ibanza.