From the Department of Biosciences and Nutrition
Karolinska Institutet, Stockholm, Sweden

ER SUBTYPE-SPECIFIC REGULATION OF ESTROGEN SIGNALING

Wen Cai

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To my family

ABSTRACT

The estrogen receptor (ER) isoforms, ERα and ERβ, mediate the physiological functions of the female hormone estrogen. They share high degree of similarity but also show significant differences in many aspects, such as cell and tissue distribution. The activities of ER are tightly regulated by different factors at multiple levels. The biological action of estrogen is the result of a balance between ERα and ERβ activities; and disruption of this balance leads to various health disorders. Understanding the mechanisms of ER subtype specific regulation is critical to understand estrogen signaling in health and disease. In this thesis, we study the possible mechanisms by which estrogen signaling could be regulated in an ER subtype-specific manner.

Intracellular ERα and ERβ protein levels or ERα/ERβ ratio are important determinants for estrogen action. Cellular regulatory factors could affect estrogen signaling by direct regulation of intracellular levels of individual ER isoforms. In ***paper I*** we demonstrate that the circadian system specifically modulates the expression of ERβ. We show that circadian regulators are recruited to the E-box enhancer in ERβ promoter and regulate the transcription of ERβ. Thus, the intracellular level of ERβ as well as ERα/ERβ ratio vary significantly at different times of the day.

Cellular factors could affect the transcription also by regulating ERs on the estrogen target gene promoter. In ***paper II***, we show that the hepatitis B virus X protein associated protein 2 (XAP2) also known as ARA9 influences estrogen signaling by interacting with ERα on the promoter region of ER-target gene in breast cancer cells. Through this mechanism, XAP2 regulates the estrogen target gene transcription in an ER subtype-specific manner, as XAP2 inhibits ERα, but not ERβ-mediated transcription.

In ***paper III*** we identify a novel epigenetic mechanism under the ER subtype-specific regulation of gene expression. We show that ERβ, but not ERα, is essential in maintaining the unmethylated state of one specific CpG in glucose transporter 4 (Glut4) promoter. This CpG is part of a specificity protein 1 (Sp1) binding site and this regulation is important for normal Sp1 recruitment and Glut4 transcription in adipocytes.

In conclusion, we have identified three novel pathways in mediating the ER subtype specific regulatory effects.

LIST OF PUBLICATIONS

I. **Wen Cai**, Juliette Rambaud, Michèle Teboul, Ingrid Masse, Gerard Benoit, Jan-Åke Gustafsson, Franck Delaunay, Vincent Laudet and Ingemar Pongratz. *Expression levels of estrogen receptor beta are modulated by components of the molecular clock*. Mol Cell Biol. 2008 Jan;28(2):784-93.

II. **Wen Cai***, Tatiana V. Kramarova*, Petra Berg, Marta Korbonits, Ingemar Pongratz. *The Immunophilin-Like Protein XAP2 Is a Negative Regulator of Estrogen Signaling through Interaction with Estrogen Receptor α*. PLoS ONE 6(10): e25201. 2011 Oct.
* Contributed equally

III. Joëlle Rüegg, **Wen Cai**, Mohsen Karimi, Nimrod B. Kiss, Elin Swedenborg, Catharina Larsson, Tomas J. Ekström, and Ingemar Pongratz. *Epigenetic Regulation of Glucose Transporter 4 by Estrogen Receptor β*. Mol Endocrinol, December 2011, 25(12).

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LIST OF ABBREVIATIONS

AF	Activation function
AhR	Arylhydrocarbon receptor
AIP	Aryl hydrocarbon receptor-interacting protein
AR	Androgen receptor
ARA9	Aryl hydrocarbon receptor-associated protein 9
ARNT	Arylhydrocarbon receptor nuclear translocator
bHLH	Basic helix-loop-heix
BMAL1	Brain and muscle ARNT-Like protein1
CARM1	Coactivator-associated arginine methyltransferase 1
CBP	CREB-binding protein
CCG	Circadian controlled gene
ChIP	Chromatin immunoprecipitation
CLOCK	Circadian locomotor output cycles kaput
CNS	Central nerve system
CRY	Cryptochrome
DBD	DNA binding domain
DES	Diethylstilbestrol
DPN	Diarylpropionitrile
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptor
ERE	Estrogen response element
ERRα	Estrogen-related receptor α
FASPS	Familial advanced sleep phase syndrome
FIPA	Familial isolated pituitary adenoma
GEF	Glut4 enhancer factor
GTF	General transcription factor
Glut	Glucose transporter
GREB1	Gene regulated in breast cancer protein 1
HSP	Heat shock protein
KO	Knockout
LBD	Ligand binding domain
LH	Luteinizing hormone
LXR	Liver X receptor
MEF	Mouse embryonic fibroblast
MEF2	Myocyte enhancer factor 2
NcoR	Nuclear receptor co-repressor
NIDDM	Non-insulin-dependent diabetes mellitus

NR	Nuclear receptor
PAS	PER-ARNT-SIM
PCB	Polychlorinated biphenyls
PER	Period
PPAR	Peroxisome proliferator-activated receptor
PPIase	Peptidyl-prolyl cistrans isomerases
PPT	Propylpyrazoletriol
RAR	Retinal acid receptor
ROR	Retinoid-related orphan receptors
SCN	Suprachiasmatic nuclei
SERM	Selective estrogen receptor modulator
SMRT	Retinoic acid and thyroid hormone receptor
Sp1	Specificity protein 1
SRC	Steroid receptor coactivator
TF	Transcription factor
TPR	Tetratricopeptide
TR	Thyroid hormone receptor
VDR	Vitamin D receptor
WT	Wildtype
XAP2	Hepatitis B virus X protein associated protein 2
ZT	Zeitgeber time

1 INTRODUCTION

1.1 Hormones and hormone signaling

Hormones are chemical messengers produced and secreted by endocrine glands. They are released directly into the blood and coordinate the functions of different organs throughout the body. Hormone-regulated biological activities are crucial in maintenance of the physiological homeostasis and regulating physiological processes including growth and development, metabolism, electrolyte balances, and reproduction [1]. Based on their chemical structures, hormones can be divided into three classes: peptide hormones such as insulin and growth hormone, amino acid derivates such as tryptophan, as well as lipid-derived hormones such as steroid hormones.

Hormones initiate a biological response by binding to their specific receptors in the target cell. In the case of steroid hormones, the hormone can cross the cell membrane and bind to the intracellular receptor. This triggers a conformational change in the receptor and results in an activated transcription factor that mediates a cellular response.

1.2 Estrogen receptors

The female sex hormone estrogen is one of the first isolated steroid hormones. Like other steroid hormones, estrogens exert their biological actions by binding to the specific intracellular receptors, namely estrogen receptors (ERs). ER-mediated estrogen signaling is essential for the normal female reproductive function by controlling the development of female secondary sexual characteristics, regulation of gonadotropin secretion for ovulation as well as preparation of tissues for progesterone response. However, the importance of estrogens for man and non-reproductive processes has also been emphasized. For example, estrogens have been identified to play critical roles in maintenance of bone mass, regulation of lipid synthesis and the regulation of insulin responsiveness [2,3,4]. Disregulation of estrogen signaling may promote the pathological processes of various diseases, such as reproductive disorders [5], cancer [6,7], metabolic syndrome [8], behavioral disorder [9] and so on.

1.2.1 Nuclear receptor superfamily

ERs belong to the nuclear receptor (NR) superfamily, a family of ligand activated

transcription factors. A large number of nuclear hormone receptors have been identified, including steroid hormones receptors (e.g. ER, androgen receptor AR), thyroid hormone receptors (TRs), retinal acid receptors (RARs) and vitamin D receptors (VDRs), as well as different "orphan receptors" with unknown natural ligand (e.g. Liver X receptors LXRs, peroxisome proliferator-activated receptors PPARs) [10,11].

NRs are key players in diverse biological functions, including homeostasis, reproduction, development and metabolism. NR biological activities are often controlled by small compounds known as ligands. These compounds that can be modified by drug design make NR interesting drug targets. Thus, NRs have drawn great interest as potential drug targets in therapies of cancer, cardiometabolic diseases as well as disorders in central nervous system [11,12].

1.2.2 Structures

The ERs share considerable structural homology with other NRs [13]. The ERs are comprised of six distinct functional domains labeled A/B, C, D, E and F (**Fig 1**).

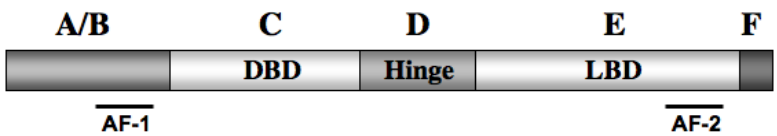

Figure 1. Schematic structural representation of the human ER.

The amino-terminal A/B domain contains activation function AF-1. The C domain is called DNA binding domain (DBD) and mediates sequence specific DNA binding activity. The DBD of ER is well conserved and displays the highest degree of sequence similar to other NRs. The DBD contains two domains, a D-box and a P-box where the "P box" is responsible for specific interaction with DNA on the typical estrogen response element (ERE) containing AGGTCA motifs, whereas the 'D box' is serves as a dimerization interface [14]. The D domain works as a flexible hinge between the C and E domain and also includes a nuclear localization signal. The E domain, which is also referred as the ligand-binding domain (LBD), contains the second activation function AF-2. The LBD binds to the ligand and transmits the signal to the transcription complex; it is also involved in receptor dimerization. AFs function as the binding sites for other regulatory proteins such as co-factors [15,16]; Synergistic effects between

ER AF1 and AF2 on estrogen-induced transcription mediated by certain co-factors have been reported [17]. The function of the C-terminal F domain is not yet fully understood.

1.2.3 Ligands

There are three endogenous estrogens, namely estrone (E1), estradiol (E2), and estriol (E3) (**Fig 2A**). As the predominant estrogen form in premenopause females, estradiol is the most potent estrogen produced in the body and both ER isoforms bind estradiol with high affinity; estrone and estriol are weaker agonists on ERs [18] and are the primary estrogens during menopause and pregnancy, respectively. The biological role of these compounds is however currently unclear. The developing follicles and ovaries are the principle source of estrogen; however, many other tissues and cells have also shown the capacity to synthesize estrogens. Importantly, in postmenopausal women and men, estrogen could be produced in a number of extragonadal sites such as liver, breast and adipose tissue [2,3,19].

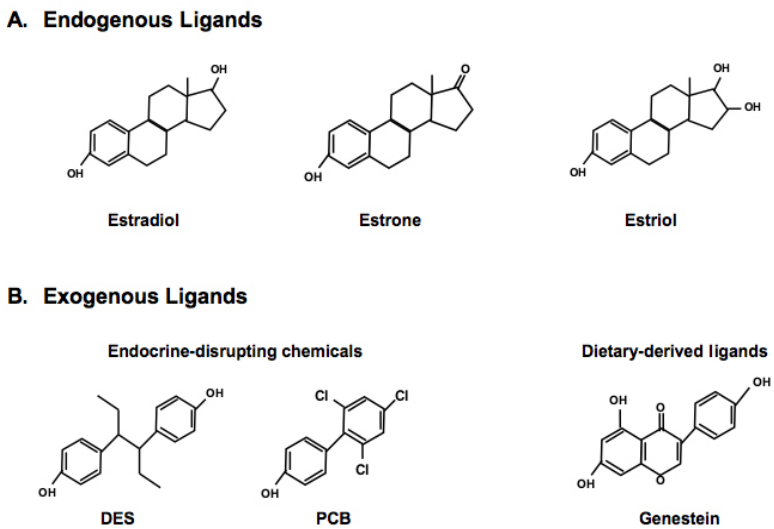

Figure 2. Structures of ER ligands. **(A)** The three endogenous estrogens: estradiol, estrone and estriol. **(B)** Exogenous compounds that bind to ER and mimic the effect of estrogen: endocrine-disrupting chemicals such as DES and PCB; and dietary-derived ligands such as genestein.

In addition, there are diverse exogenous compounds that bind to ER and mimic the effect of estrogen (**Fig. 2B**). Some of them are man made compounds, for example, certain endocrine-disrupting chemicals, such as diethylstilbestrol (DES), polychlorinated biphenyls (PCBs), alkylphenols, phthalates, and parabens, have been shown to have estrogenic effects and are known as xenoestrogens [20]. The exogenous ER ligands could also be dietary-derived, for example, a range of plant products, namely phytoestrogens, could induce estrogenic activities in mammals. The main classes of phytoestrogens include the flavonoids, lignans and coumestans, which are abundant in nuts, oilseeds and soy products [21].

1.2.4 Co-factors

The AF-1 and AF-2 domains of ERs as well as other NRs mediate recruitment of a multitude of cellular regulatory co-factors which affect the rate of ER target gene transcription. As a crucial step in the process of ER transcriptional activation, regulations by co-factors are important for normal functions of ER. Thus, the activities and cellular concentrations of co-factors can have a great impact on ER-controlled physiological and pathological processes.

Co-activators represent a group of cellular factors that promote the transcriptional activity of the receptors in the presence of ligand. They bind to NRs receptors through Leu-Xaa-Xaa-Leu-Leu motifs (NR boxes). Co-activators consist of different classes of proteins, for instance, steroid receptor coactivator (SRC/P160) family, CREB-binding protein (CBP), coactivator-associated arginine methyl-transferase 1 (CARM1) and so on. These proteins have diverse structures and facilitate the transcription through different mechanisms such as histone acetylation, histone methylation, or ubiquitination [22,23,24].

Alternatively, Co-repressors such as silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) and nuclear receptor co-repressor (NcoR) are proteins that mediate a repressive effect of the receptors. Recent studies showed that co-repressors can be recruited by both agonist- and antagonist-bound ERs, however, the role of co-repressors for ER is not established as well as that of coactivators [25,26].

1.2.5 ER signaling

Two different modes of ER signaling mechanisms have been shown: the genomic action and the no-genomic action (**Fig. 3**).

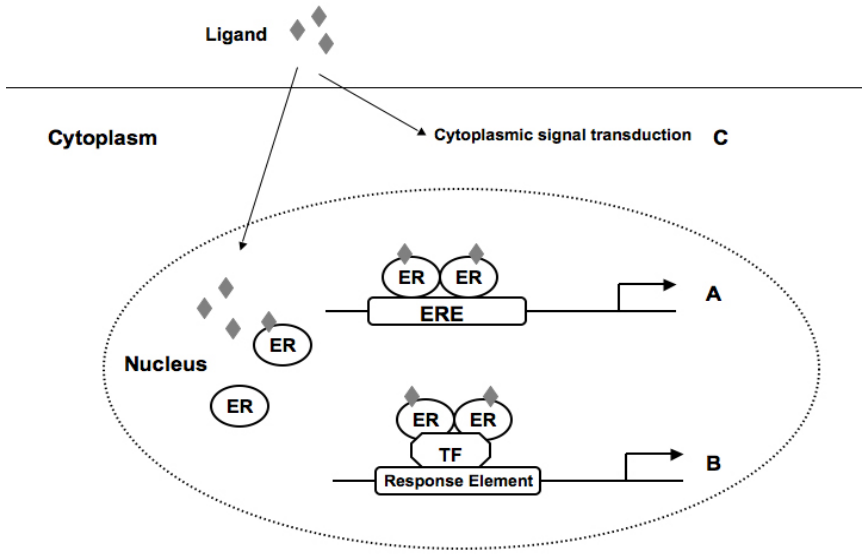

Figure 3. Mechanism of ER signaling. ER regulates gene expression through **(A)** ligand-activated ER binds to ERE; **(B)** ligand-activated ER binds a response element via another transcription factor (TF); **(C)** non-genomic mode.

The classical genomic ER signaling involves ER binding to specific DNA enhancer or promotor elements and thus regulation of expression of certain sets of genes (**Fig. 3A**). In the absence of ligand, ER is present in an inactive form associated with cellular chaperones like heat shock proteins (HSP90). In the presence of ligand the ER is activated and dissociate from the chaperones. Activated ER is phosphorylated and binds as homodimers or heterodimers to EREs in the promoter region of a target gene. The essential ERE sequence was determined to have the consensus inverted repeat 5'-GGTCAnnnTGACC-3'. ER-DNA interaction leads to a change of the local DNA structure and open up the DNA, which induces the recruitment of general transcription factors (GTFs) to the promoter and initiates the transcription of the target gene [27,28,29,30].

There is also another type of genomic ER signaling that involves indirect ER-DNA binding (**Fig. 3B**). In this pathway, activated ERs regulate target gene in an ERE independent manner by interacting with other DNA-binding transcription factors. For instance, ERs have been shown to activate genes via specificity protein 1 (Sp1) binding site by interacting with the Sp1 transcription factor [31,32].

The non-genomic ER signaling is characterized by the rapid ligand response and possibly mediated by membrane-associated ER (**Fig. 3C**). It has been suggested that these effects involve the activation of MAPK/ERK pathway in cytoplasm [33,34]. However, the precise role of membrane bound ER is currently unclear.

1.3 ER subtypes

There are two identified ER subtypes. The first ER, ERα (ESR1, NR3A1), was cloned from MCF-7 breast cancer cell line in 1985 [35]. ERα was regarded as the only estrogen mediating receptor until a novel ER isoform, ERβ (ESR2, RN3A2), was cloned from rat prostate library in year 1996 [36]. Both ERα and ERβ are indispensable in mediating physiological functions of estrogens, however, they often exert distinct effects. In many aspects, these two ER subtypes share large degree of similarities but also show significant differences [37,38,39].

1.3.1 Expression patterns

Although both ERα and ERβ are widely distributed throughout the body, they display distinct expression patterns in different tissues and cell types. ERα is mainly expressed in ovary (theca cells), uterus, prostate (stroma), testis (Leydig cells), epididymis, breast, liver, bone, skeletal muscle, wihite adipose tissue as well as certain regions of the brain, such as pituitary and hypothalamus; whereas ERβ is expressed in ovary (granulose cells), prostate (epithelium), testis, colon, lung, bone marrow, salivary gland as well as regions in the brain including hypothalamus and cortex [40,41].

1.3.2 Structural homology

As mentioned above, both ER subtypes share considerable structural similarities with other members of the NR family of transcription factors. The DBDs of ERα and ERβ are highly homologous with 97% amino acid identity and both bind to the consensus ERE. The LBDs of two ERs share relatively high degree of homology with 56% amino acid identity, which provide a basis for binding of common and subtype-specific ligands. The N-terminal A/B domain is poorly conserved between the two ER subtypes, which display only about 20% homology. This counts for the different AF-1 activities and co-factor recruitment of ERα and ERβ [42,43].

1.3.3 Knockout animals

Through the generation of the ER knockout (ERKO) animal models there is direct evidence showing the physiological roles of estrogen signaling in both reproductive and non-reproductive systems. Phenotypes of ERα and ERβ KO (αERKO and βERKO) mice have further illustrated both the similarities and differences of these two ER subtypes in biological function.

Reproductive malfunctions are observed following disruption of either ERα or ERβ. In αERKO mice, both males and females are infertile, whereas in ERβ KO animals, only the female display subfertility. It has been suggested that both ERs are required for efficient ovulation, whereas ERα appears to be more critical than ERβ in maintenance of testicular structures and the somatic cell function required for successful sperm maturation [44,45].

It has been shown that the pubertal growth of the epithelial ductal rudiment of mammary gland is impaired in the αERKO female, which is due to the abnormal function of αERKO pituitary; in contrast, adult βERKO females display a fully developed mammary gland with ductal network similar to the wild type, suggesting that ERα, but not ERβ, is required for the structural and functional development of the mammary gland [44]. Moreover, higher fasting blood glucose levels and impaired glucose tolerance were observed in αERKO but not in βERKO mice, implying a more important role of ERα in mediating the estrogen effects on glucose metabolism [46].

The observed phenotypes of βERKO mice reveal crucial roles of ERβ in development and function of central nerve system (CNS), bone, hearing as well as respiratory system. For instance, at the late embryo stage, the brains of βERKO mice are smaller than those of littermate controls, especially in the cerebral cortex, suggesting that ERβ is a key player in brain morphogenesis [47]. It has also been shown that βERKO mice were deaf at 1 year of age with abnormal inner ear morphology such as the absence of hair cells [48]. Furthermore, there is study showing that βERKO mice have systemic hypoxia caused by abnormal lung structures including larger alveoli and reduced elastic recoil [49].

1.3.4 Ligand affinities

E2 binds the two ER subtypes with similar affinity; however, other ligands or compounds may differ considerably in their binding affinities for ERα and ERβ. For instance, E1 has been shown to display higher affinity for ERα, whereas E3 binds preferentially with ERβ [50]. The functional consequence of this difference

is currently unclear.

Phytoestrogens and xenoestrogens show different degree of affinities for ERα and ERβ [40]. For example, genistein, a soy isoflavone, manifests a much higher affinity for ERβ than for ERα [51], whereas another well-known phytoestrogen, raloxifene, displays an ERα-selective affinity [52]. It has also been reported that the endocrine disrupting chemicals HO-PCBs show greater binding preference for ERβ over ERα [53].

Two synthetic non-steroidal compounds, propylpyrazoletriol (PPT) [54] and diarylpropionitrile (DPN) [55] have been identified and characterized as selective agonists for ERα and ERβ, respectively. PPT is approximately 1000-fold more potent as an ERα agonist compared to ERβ and has a 400-fold preference towards ERα in its binding affinity, whereas DPN shows a 70-fold higher relative binding affinity for ERβ than for ERα [56].

The selective estrogen receptor modulators (SERMs) refer to a group of synthetic molecules that have estrogen-like effect in some circumstances but act as anti-estrogens in the others, depend on the target tissues or ER-subtype. Certain SERMs have shown great pharmaceutical value since they could activate the benefit effect of ERα/β, but inhibit the undesired side effect of ERα/β. Tamoxifen was the first SERM that has been used in the clinical treatment [57]. Given that it displays an antagonistic effect on the breast while functioning as an agonist in uterus and skeletal tissue, tamoxifen is now widely used in breast cancer therapy.

1.3.5 Co-factor selectivity

As other NRs, activation of ER signaling requires interactions between ER with different co-factors. Besides the ligand-specific and tissue-specific regulation of ER-co-factor interaction, ERα and ERβ also show affinity preferences for particular co-activators. For instance, classical p160 co-activators, especially SRC3, bind ERα with much higher affinity [58]; on the other hand, the novel ER co-factor arylhydrocarbon receptor (AhR) nuclear translocator (ARNT), although has been identified to co-activate both ER subtypes, preferentially facilitates the transcriptional activity of ERβ [59].

Given that AF-1 domain serves as the binding surface for various co-factors, the poor sequence homology between ERα and ERβ in the AF-1domain could be responsible for the differential affinity preferences of co-factors for either ERα or ERβ. Furthermore, it's also been shown that ERα and ERβ utilize different

LXXLL motifs for their interaction with p160 proteins [58].

It is also noteworthy that the ER subtype-selective co-activating could have significant physiological importance. For example, SRC-3 amplification has been found in ERα-positive breast cancers [60,61], whereas AhR-ARNT ligand dioxin shows more potent effect in disruption of ERβ signaling [59].

1.3.6 Roles in human diseases

Different disease patterns have been noticed in male and female, as well as in premenopausal and postmenopausal females, implying the involvement of estrogen signaling in human diseases. To date, identified estrogen influenced diseases include cardiovascular diseases, metabolic syndrome, tumors, osteoporosis, neurodegenerative diseases, mode disorders, autoimmune diseases and so on [62,63].

ERα and ERβ exert opposing effects in certain diseases. For example, several studies have demonstrated that ERα primarily mediates the tumorigenic effects of estrogens. Lifetime exposure to estrogen ligands and high estrogen levels and thus high ER transcriptional activity represent a risk factor for developing tumors in breast [6], endometrial [64], ovarian [65] pituitary [66] and thyroid tissues [7]. In contrast, ERβ has been shown to possess a tumor suppressive effect in tissues such as the prostate [67] and colon [68].

1.3.7 Regulation of ER-subtype activity

Estrogen signaling is regulated by a complex multi-level procedure involving diverse factors. ER could display distinct activities and functions in different cellular environments (**Fig. 4**).

Tissue-specific distribution of ER implies that regulatory factors, which influence the expression of ER, could act as key modulators of ER activity and estrogen signaling (**Fig. 4A**). This regulation could occur at different levels, including DNA modification, transcription, post-transcription and translation. However, the transcription regulation that controls the initiation of the ER gene transcription is often the principal step.

Ligand binding is the first essential regulatory step in the ER activation process (**Fig. 4B**). Different ligands induce distinct conformational changes in ER [69]. The structure, concentration and affinity of the ligands all have been shown to

have great influence on ER transcriptional efficiency.

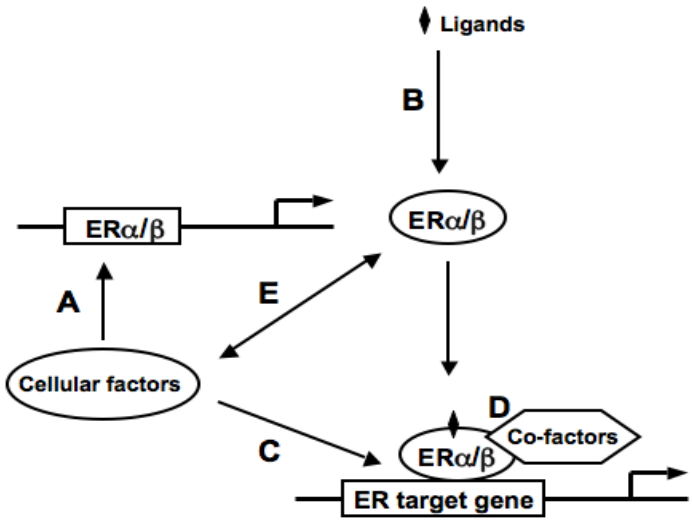

Figure 4. Regulation of ER activity is a multi-level procedure involving diverse factors such as: **(A)** cellular factors that influence the expression of ER; **(B)** ligand binding; **(C)** cellular factors that affect the accessibility of the target gene promoter; **(D)** the expression and activity of co-factors; **(E)** other factors and mechanisms.

Furthermore, as a transcription factor (TF), ER exhibits transcriptional activity by binding to the target gene promoter. Thus, cellular factors that affect the accessibility of the target gene promoter could also have significant impact on ER-mediated biological activities (**Fig. 4C**).

In addition, the expression and activity of co-factors (**Fig. 4D**) as well as other ER associated proteins such as Sp1 could also be important determinants of ER-mediated transcription through different mechanisms (**Fig. 4E**).

1.3.8 ERα / ERβ balance

As mentioned above, ERα and ERβ have distinct functions and modulate different effects. Actually, the two ER subtypes are often antagonistic towards each other. Biological action of estrogen is the result of a balance between ERα and ERβ mediated estrogen signaling; disruption of the ERα/ERβ balance could lead

to various health disorders [18,70].

The opposing roles of the two ER subtypes in regulation of cell proliferation have been observed. ERα has been shown to promote the cell proliferation in various tissues including breast, uterus as well as developing prostate; whereas the role of ERβ in these tissues is rather to inhibit proliferation and induce differentiation. This provides an important message for tumor biology since cell hyper-proliferation is a crucial process in tumorigenesis. Possibly an important role of ERβ is to protect against ERα-induced cell proliferation. Actually, an increased ERα/ERβ mRNA ratio in estrogen-induced tumorogenesis has been reported in breast, ovary, colon, and prostate cancers [71,72].

The relationship between ERα and ERβ are often mentioned as an "yin/yang balance", which describes the fact that two ER subtypes have both overlapped and differential functions, cooperate with each other whereas at the same time restrain against each other. This concept could bring a new insight for both therapy method and drug development in estrogen-associated diseases.

1.4 Circadian system

Circadian rhythm is an internal biological clock existing in almost all living organisms. It integrates and regulates a variety of biological processes according to a roughly-24-hour period [73]. In mammals, including humans, normal daily rhythm is important in maintaining physiological functions like the sleep-wake cycle, body temperature, feeding behavior, glucose homeostasis, drug and xenobiotic metabolism [74]. Disruption of the normal circadian rhythm (e.g. shift work) is associated with health disorders such as cancer, mood disorders, metabolic syndrome, cardiovascular diseases, and gastrointestinal disease as well as reproductive malfunctions [75,76].

1.4.1 The central and peripheral clocks

The central circadian pacemaker in mammals is located in the suprachiasmatic nuclei (SCN) of the ventral hypothalamus [77]. This "master" internal clock is synchronized primarily by the daily light-dark cycle and coordinates the rhythms of multiple local clocks in peripheral tissues and cells through both neuronal and hormonal signals [74,78]. Lesions in the SCN region result in a loss of circadian rhythmicity in both behavior and hormone secretion [79,80].

On the other hand, although the peripheral clocks require the inputs from the

master clock in order to sustain the synchrony, these clocks are also adjusted by non-photic time cues including feeding time, hormones, growth factors and metabolites such as glucose [81]. It has been shown that most of the circadian controlled genes (CCGs) are expressed in a tissue-specific manner, demonstrating the importance of the local clock in regulation of tissue-specific biological functions [82,83]. Moreover, circadian rhythms have been identified even in cultured cells, which can be synchronized by treatment of high concentration of serum, hormones or growth factors [84,85].

1.4.2 Circadian genes

The circadian system is regulated by a set of clock genes that highly conserved through evolution. Disruption of clock gene expression by mutation or KO could results in profound disturbance of the normal circadian rhythmicity.

The positive circadian regulators include the circadian locomotor output cycles kaput (CLOCK) and the brain and muscle ARNT-like protein1 (BMAL1). Both CLOCK and BMAL belong to the helix-loop-heix (bHLH)-PER-ARNT-SIM (PAS) superfamily. It has been reported that CLOCK mutant mouse heterozygotes displayed an abnormally long period of daily activity; and homozygotes generate arrhythmicity after several days in constant darkness [86]. BMAL1 is also known as ANRT3 or MOP3, BMAL1 KO mice lost all circadian rhythmicity in constant darkness and showed impaired locomotor activities (whole body movement) in light-dark cycles [87].

There are also negative regulators of circadian system, namely Period (PER) and Cryptochrome (CRY). PERs (PER1 and PER2) are also members of PAS family, mutations in PER2 gene has been identified in patients suffering from familial advanced sleep phase syndrome (FASPS) [88]. CRYs (CRY1 and CRY2) represent a class of blue light-sensitive flavoproteins. Interestingly, in constant darkness, CRY1 mutant mice display a shortened circadian period, whereas CRY2 mutant mice had a prolonged circadian period, only double-mutant mice showed instantaneous arrhythmicity [89].

1.4.3 Molecular oscillators

Molecular circadian oscillator is driven by a transcription/translation feedback loop that leads to recurrent rhythms in the RNA and protein levels of key clock components [90] (**Fig. 5**)

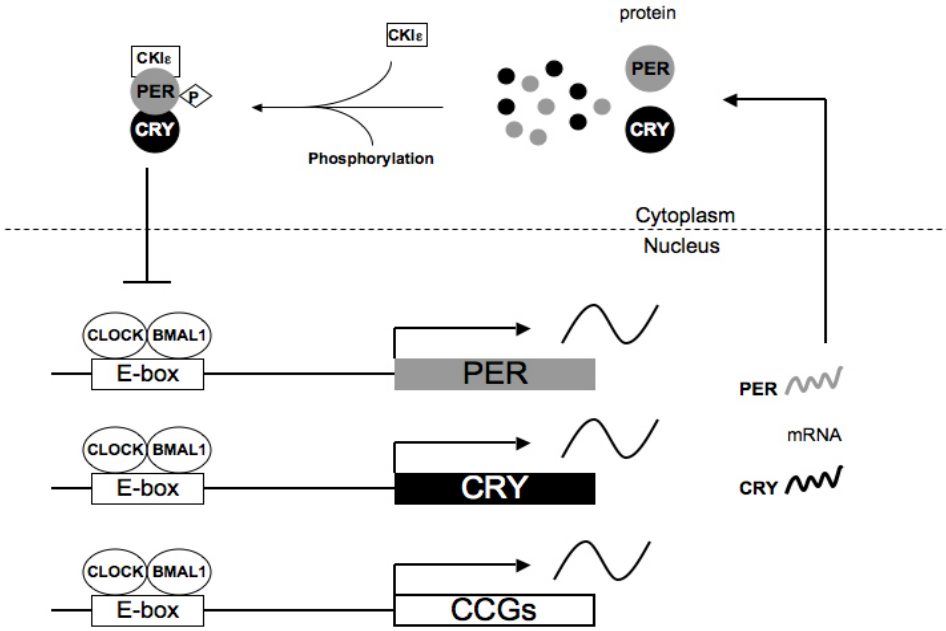

Figure 5. Molecular circadian oscillator is driven by a transcription/translation feedback loop.

The positive arm of the rhythmicity consists of CLOCK and BMAL1, which form heterodimer and bind to the E-box motifs within the promoter regions of CCGs and generate the transcription of these genes. Among these CCGs, there are negative circadian regulators PER and CRY. Accumulated PER and CRY proteins form in turn negative regulatory complexes by binding with each other as well as casein kinase CKIε. PER-CRY-CKIε is then phosphorylated and translocates to the nucleus. By interacting with the CLOCK-BMAL1, the negative regulators inhibit transcriptions of their own genes and other CCGs [77,81,91] and complete the negative arm of the loop. Furthermore, it has been shown that CLOCK has enzymatic histone acetyl-transferase activity could directly control the chromatin remodeling of target gene [92].

In addition, besides the main feedback loop, there is also another feedback loop known as “the second feedback loop” or “REV/ROR/BMAL1 loop”, which involves the orphan NRs retinoid-related orphan receptors (RORs) and REV-ERB (α and β). The expression of REV-ERB is regulated by CLOCK-BMAL1. Both ROR and REV-ERB bind to the specific response elements RORE in the

promoter region of BMAL1 gene and display opposite effects: ROR activates the transcription, whereas REV-ERB represses the transcription. Thus, the rhythmic expression of BMAL1 is the result of competition between REV-ERBs and RORs at ROREs. This mechanism contributes possibly to the robustness of the circadian oscillations [93].

1.4.4 Circadian clock and reproduction

Accumulating evidences suggest a clear connection between circadian rhythm and reproduction. The circadian system regulates the reproductive functions by influencing the timing of puberty, sex hormone secretion, ovulation and mating activities. It has been shown that CLOCK mutant female mice displayed malfunctions in reproductive system such as irregular estrous cycles, lack a coordinated luteinizing hormone (LH) surge on the day of proestrus, exhibit increased fetal reabsorption during pregnancy, and have a high rate of full-term pregnancy failure [94]. Moreover, infertility has been observed in both male and female BMAL1 KO mice by several labs [95,96]. In humans, despite the fact that only few studies have been performed, there is evidence showing associations between female shift workers and reproductive malfunctions such as irregular menstruation, increased risk of spontaneous abortion and preterm birth [97,75].

1.4.5 Circadian clock and NRs

The crosstalk between the circadian clock and the NR signaling pathway exists at multiple levels. First, NRs like ROR and REV-ERB could function as core players in the molecular pacemaker (as described above); Second, It has been known for decades that plasma levels of NR ligands such as glucocorticoids and aldosterone display daily rhythms; in addition, the expressions of several NRs have been identified to be clock-regulated and display a circadian pattern. These NRs include REV-ERB, estrogen-related receptor α (ERRα), PPARα and TRα. Furthermore, clinical observations imply an interaction between circadian system and ERs, for example, disturbance of circadian genes have been shown in breast cancer and a correlated decrease of PER1 and ERβ in colorectal tumors is also been reported [98,99]. However, although it has long been known that circadian clock could influence variety of physiological processes, the exact mechanism behind the regulation is not clear yet. One possible mechanism could be that circadian clock modulates physiological pathways by regulating the expression of certain CCGs, which are key factors in these pathways. NRs could be such factors that transmit the circadian rhythm from the core oscillator to physiological outputs [100].

1.5 XAP2

The Hepatitis B virus X protein-associated protein 2 (XAP2), also known as aryl hydrocarbon receptor-associated protein 9 (ARA9) or aryl hydrocarbon receptor-interacting protein (AIP), is a 37 kD Hsp90-associated protein that belongs to the immunophilin family of proteins. XAP2 was initially identified as a specific AhR co-chaperone however later studies have shown that other signal transduction proteins are regulated by XAP2. Although XAP2 is ubiquitously expressed, the expression levels of XAP2 vary considerably in different tissues, with highest levels of expression in the spleen and thymus and low expression levels in the liver, kidney and lung [101,102,103]. XAP2 shares structural homology with other immunophilins, the protein contains a peptidyl-prolyl cistrans isomerases (PPlase)-like domain and three tetratricopeptide repeats (TPR). Each of the three TPR motifs of XAP2 consists of two α-helices forming an antiparallel amphipathic structure that mediate intra- and inter-molecular interactions with other proteins, such as Hsp90 [104].

1.5.1 Traditional functions of XAP2

Although structurally sharing a significant homology with immunophillins, XAP2 does not function as other immunophinlins to mediate the effects of immunoreppressant drugs [104]. It was originally identified as a negative regulator of the hepatitis B virus X-associated protein [103]. Later, the role of XAP2 as an Hsp90-associated protein in regulating AhR was intensively studied. It has been shown that low levels of XAP2 expression enhanced human AhR signaling, whereas high-level expression of XAP2 blocked AhR signaling in a synthetic yeast model system [105]. In mammalian cells, XAP2 interacts specifically with AhR-Hsp90 complex and induces the cytoplasmatic redistribution of AhR [103,106]. In addition, XAP2 stabilizes the AhR in the absence of ligand by inhibiting AhR ubiquitination and proteosomal degradation [107].

1.5.2 Novel roles of XAP2

The sequence of XAP2 protein is highly conserved through evolution. Moreover, XAP2 KO mice display cardiac defects and 100% embryonic lethality [108]. This evidence suggests that XAP2 could be involved in a wider range of biological processes. Remarkably, increasing numbers of XAP2 client regulated proteins with diverse physiological functions have been identified as interacting partners of XAP2. These proteins include not only chaperone proteins and viral proteins, but also novel XAP2 partners such as G proteins, transmembrane receptors,

mitochondrial import receptors as well as several NR family members [109].

Recently, the tumor suppressive role of XAP2 has drawn considerable interest in clinical studies. Germline mutations in XAP2 genes have been identified in both familial and sporadic pituitary tumor patients [110]. Clinical data showed that about 30% of all familial isolated pituitary adenoma (FIPA) families and 50% of acromegaly families carry a mutation in the XAP2 gene [111]. Patients with XAP2 mutations predispose to young-onset pituitary tumours, most often to growth hormone-or prolactin-secreting adenomas [104]. The molecular mechanisms behind the tumor suppressive-activity of XAP2 have however not been clarified yet. One possibility is that the XAP2 interacts with regulatory factors and thus modulates pathways involved in tumor development as well as other pathological processes. Interestingly, several studies have showed that estrogen could induce the formation and development of pituitary tumor [112,113], suggesting the involvement of ER-regulated signaling pathways in pituitary tumor pathogenesis. In addition, Naves and co-workers reported precautious puberty in a one-year-old female XAP2 mutation carrier [109], implying a modified ER signaling in XAP2 mutated individuals.

1.5.3 XAP2 and NRs

Resent studies have demonstrated a physical and functional role for XAP2 in regulation of certain members of the NR superfamily.

In year 2003, Sumanasekera et al. reported that XAP2 could interact with PPARα and repress its activity in mouse liver [114].

In addition, Froidevaux et al. in 2006 indentified XAP2 as a novel partner for TRβ1. They demonstrated TR-XAP2 interactions were TR isoform specific and could be enhanced by T3. They also shown that knockdown of XAP2 protein level by siRNA could influence the stability of TRβ1 in vitro and abrogated the TRβ1-mediated activation of hypothalamic TRH transcription in vivo. However, their experiments suggest that XAP2 doesn't seem to affect the subcellular localization of TRβ1 [115].

Later, in 2009, another NR, GR, has been identified to be XAP2 asscociated. In mammalian cells, XAP2 has shown to interact with GR through Hsp90 and inhibits GR signaling by delaying the nuclear translocation of lignd-bound GR [116].

1.6 DNA methylation

1.6.1 Epigenetic regulation

Epigenetic regulation refers to the heritable modifications in gene expression and function that occur without a change in the DNA sequence. In mammals, the epigenetic modifications include DNA methylation, post-translational modifications of histone proteins and non-coding RNAs. By determining the expression pattern of genes, epigenetic regulation plays crucial role during growth and development. Epigenetic homeostasis in cells is essential in maintenance of normal cellular functions [117,118].

1.6.2 DNA methylation

DNA methylation is the only known epigenetic modification of DNA in mammals [119] and is an inhibitory factor of gene expression [120]. The predominant form of DNA methylation is methylation of cytosine at position C5 in CpG dinucleotides in the target promoter, which could either inhibits the binding of transcriptional activating proteins or recruits methylated DNA-binding factors to the promoter [121]. The CpG island is a DNA fragment with high frequency of the CG sequence. CpG methylation could induce histone deacetylation, chromatin remodeling and thereby lead to a gene silencing [122]. In addition, importance of non-CpG methylation has been more and more emphasized. For instance, cytosine-5 methylation at CpA and CpT has shown to present significantly in embryonic stem cells [123]; also, 5-methylcytosine can be converted to 5-hydroxymethylcytosine by the ten-eleven translocation (TET) methylcytosine dioxygenases[117].

Furthermore, in human and other mammals, demethylation and remethylation occur genome-widely during the early embryonic development [124,125,126]. Resently, Tet-catalyzed oxidation followed by decarboxylation has been proposed to be a possible mechanism of the DNA demethylation [127,128].

1.6.3 DNA methylation and diseases

DNA methylation is crucial in regulating many cellular processes; defects of normal DNA methylation are correlated with various human diseases. In cancers, hypomethylation in genomes of cancer cells occurs at the very early stage of carcinogenesis and has shown to correlate with disease severity and metastatic potential [119,129,130,131]; methylation status in skeletal muscle from type 2

diabetes differs significantly from normal glucose-tolerant volunteers, suggesting a role of DNA methylation in metabolic disorders [132]; Moreover, DNA methylation is also associated with autoimmuno diseases such as systemic lupus erythematosus, it has been suggested that the genomes of T cells in these patients are globally hypomethylated, which result in the autoantibody response [119].

1.7 Glut4

Glucose transporters (Gluts) represent a family of proteins that responsible for the glucose uptake into cells by transporting glucose cross the plasma membranes. The Glut family consists of several isoforms (Glut1-Glut5), which are expressed by various cell types and play essential roles in maintaining glucose homeostasis. The Glut4 isoform is the insulin-response glucose transporter in skeletal muscle, cardiac muscle and adipose tissue [133,134].

1.7.1 Functions of Glut4

Glut4 functions as the major mediator of insulin-stimulated glucose uptake into striated muscle and adipose tissue. In the low-insulin condition, Glut4 is stored in intracellular pools. Experiments have shown that rise of blood glucose level (e.g. after food intake) will induce insulin secretion, which leads to the translocation of Glut4 storage vesicles to the plasma membrane. Glut4 proteins will then be inserted into the cell surfaces as these Glut4-containing vesicles fuse with the plasma membrane. Glut4 will subsequently promote import of glucose into for example muscle cells and subsequently a reduction in circulating glucose levels [134,135,136,137]. The major regulator of Glut4 function is insulin, which binds to the insulin receptor and induces a rapid translocation of Glut4; the number of Glut4 at the cell surface of muscle cells could also induced by contraction, depolarization, or energy deprivation [138]. In addition, other factors, such as estrogen, could also affect Glut4 function by regulating the expression level of Glut4 in different tissues [139].

Phenotypes of transgenetic animals imply an essential role of Glut4 in glucose metabolism. Glut4 KO (Glut4 -/-) mice show not only severe metabolic disorders including impaired insulin tolerance, hyperinsulinaemia, decreased levels of lactate, non-esterified fatty acids, β-hydroxybutyrate as well as fat tissue deposition, but also other disorders such as growth-retarded and cardiac hypertrophy; on the other hand, overexpression of Glut4 in skeletal muscle results in increased glucose metabolism and protects against the development of insulin resistance and diabetes mellitus [140,141,142].

In addition, pretranslational suppression of Glut4 has been shown in adipocytes from obese and non-insulin-dependent diabetes mellitus (NIDDM) patients. It is also suggested the Glut4 deficiency is a major cause of cellular insulin resistance in these patients [143]. Furthermore, exercise training increased Glut4 expression in muscle is associated with enhanced glucose tolerance Therefore, to increase the number of Glut4 in the plasma membrane in muscle and adipocytes may be a novel therapeutic method in the treatment of diseases such as NIDDM [135].

1.7.2 Transcriptional regulation of Glut4 expression

The human Glut4 promoter harbors two functional regulatory domains: the Glut4 enhancer factor (GEF) binding domain (domain I) and the myocyte enhancer factor 2 (MEF2) binding domain (domain II). The regulation of Glut4 expression is the cooperation between these two domains [144].

The transcription of Glut4 is under the control of variety of TFs. In muscle and adipose tissue, transcriptional activating factors for Glut4 expression include MEF2, GEF, MyoD, C/EBP-α, SREBP-1c and NR family members TRα 1 and LXRα; the repressor of Glut4 gene include tumor necrosis factor-α, dioxin, free fatty acids, nuclear factor-1 as well as NR PAPRγ[145]. In addition, Sp1 is shown to bind to the Glut4 promoter and facilitates the SREBP-1c mediated activation of Glut4 [146].

1.7.3 Glut4 and ER

Both ERα and ERβ have been suggested to regulate Glut4 expression levels [145,147]. Studies in ERα and ERβ KO mice suggest that ERβ reduces, whereas ERα enhances Glut4 protein levels in muscle and white adipose tissue [147]. This observation is strengthened by the fact that ERα, but not ERβ knockout mice show impaired glucose and insulin tolerance [8]. However, opposite effects have been shown in a hamster ovary cell line, where the selective activation of ERβ induces Glut4 expression [148]. Moreover, it has also been reported that ERβ inhibits the activity of PPARγ [149], one of the repressors of Glut4 transcription. Taken together, the roles of ERs on Glut4 activity are so far contradictory. It is possible that the ER subtype-dependent regulation of Glut4 is tissue- and cell type-specific.

1.7.4 DNA methylation and Glut4 expression

Previous experiments have shown that expression of Glut4 is also regulated

at the epigenetic level; for example, CpG methylation plays an important regulatory role in adipogenesis and glucose homeostasis. Tissue-specific level of methylation is an important determinant for the tissue-specific expression of metabolic regulation factors [150,151]. The transcription of Glut4, for example, is regulated by methylation. Studies show that demethylation on Glut4 promoter occurs during the differentiation of preadipocyte to adipocytes, methylations at certain CpG sites prevent the activation of Glut4 promoter [152]. Thus, DNA methylation may play as a mediator between environmental factors and metabolic disorders such as diabetes [132,153]. However, further studies are required to fully understand the mechanism clinical meaning of the methylation-dependent gene regulation.

2 AIMS OF THESIS

The general aim of the thesis is to characterize possible mechanisms by which the estrogen signaling could be regulated in an ER subtype-specific manner. In particular, our studies aimed at:

- Investigate the circadian impact on the expression of ER subtypes and consequent effects on estrogen signaling (**paper I**).

- Characterize the role of XAP2 on ERα- and ERβ-mediated estrogen signaling (**paper II**).

- Compare the effects of ERα and ERβ on Glut4 activity and characterize the mechanisms behind the effects (**paper III**).

3 RESULTS AND DISCUSSION

3.1 *Paper I*: EXPRESSION LEVELS OF ESTROGEN RECEPTOR β ARE MODULATED BY COMPONENTS OF THE MOLECULAR CLOCK

Overlap between circadian-regulated and ER-related physiological disorders have been observed in both reproductive and non-reproductive tissues, suggesting a link between these two transcription pathways. In this study performed in collaboration with Vincent Laudet (Ecole Normale Supérieure de Lyon) and Franck Delaunay (Université de Nice-Sophia-Antipolis), we identify a novel regulatory level of estrogen signaling where ER is controlled by the circadian clock. Remarkably, our experiments indicate that only one ER subtype, ERβ, is regulated by components of the molecular clock, whereas ERα is not under the circadian regulation.

By using Real-time PCR, we show that ERβ mRNA oscillates in WT mice lung and muscle, with a robust circadian pattern with a peak at zeitgeber time (ZT) 12. This oscillatory pattern is maintained under free-running conditions and abolished in clock deficient BMAL1 KO mice. However, there was no oscillation in ERα expression in either WT or BMAL1 KO mice tissues.

To investigate whether ERβ is under the direct regulation of the molecular circadian components, we performed transient transfection and chromatin immunoprecipitation (ChIP) experiments. Results from these experimetns indicated that CLOCK-BMAL1 heterodimers are efficiently recruited to a conserved E-box enhancer element in the ERβ promoter and mediate transcriptional activation. In addition, we showed that the repressive circadian factors PER-CRY are recruited to inhibit transcription. To identify which of the positive or the negative components of the circadian cycle are the critical components regulating ERβ expression, we performed siRNA assays and demonstrate that CLOCK or PER1 knock down leads to elevated expression of ERβ. This result suggests that ERβ circadian cycling is primarily maintained through PER1 recruitment to the ERβ promoter and that CLOCK-BMAL1 serve as a docking point for PER1 (**Fig. 6**).

Furthermore, using HC11 cells with stably transfected 3xERE reporter, we show that the expression levels of the circadian regulatory factors directly and specifically influence ERβ-mediated estrogen signaling by regulating the intracellular levels of endogenous ERβ.

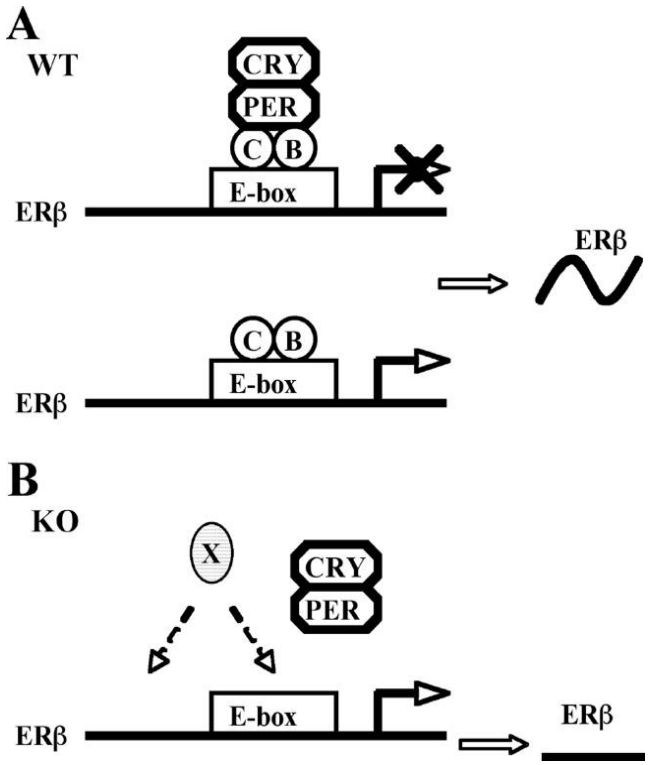

Figure 6. CLOCK:BMAL1 heterodimer mediates the circadian regulation of ERβ by binding to the E-box enhancer in ERβ promoter, but the negative regulators work as the main driver of the rhythmic expression of ERβ.
(A) In WT mice and cells, recruitment of negative circadian regulator PER:CRY causes an inhibition of ERβ expression, and the release of PER:CRY results in an up-regulation of ERβ expression induced by CLOCK:BMAL1 and other unknown activating transcription factors.
(B) In BMAL1 KO mice or CLOCK deficient cells, where CLOCK:BMAL1 heterodimer is not formed, the negative regulators are not recruited to the E-box enhancer and the expression of ERβ is induced by unknown activating transcription factors (X) and kept at a constant high level.

3.2 *Paper II*: THE IMMUNOPHILIN-LIKE XAP2 IS A NEGATIVE REGULATOR OF ESTROGEN SIGNALING THROUGH INTERACTION WITH ESTROGEN RECEPTOR α

Previous studies have demonstrated that XAP2 mutations are associated with pituitary tumors. Furthermore, there is an over-representation of reproductive tumors among patients with non-functional XAP2 expression, suggesting a possible role of XAP2 in regulating estrogen signaling in connection with tumor development. In this study, in collaboration with Marta Korbonits (Queen Mary University of London) we show that XAP2 regulates estrogen signaling in an ER subtype-specific manner, by inhibiting ERα but not ERβ-mediated transcription.

At the beginning of the study, we demonstrate that XAP2 has a negative on expression of the breast cancer marker gene pS2 as well as another ER target gene GREB1 in MCF-7 cells, suggesting a regulatory role of XAP2 in estrogen signaling. Interestingly, siRNA assays showed that knockdown of XAP2 led to an increase of 3xERE expression in only PPT and E2 treated cells, but not DPN treated cells. This result suggests that XAP2 downregulates ERα but not ERβ-mediated estrogen signaling.

Then we proceeded further to investigate the mechanism behind the XAP2-ER regulation. Although XAP2 has previously been shown to protect AhR from protein degradation by inhibiting AhR ubiquitination, reduction of XAP2 protein did not affect the intracellular protein levels of ERα. However, both our *in vivo* and *in vitro* experiments indicate that XAP2 interacts with ERα. Using mutated forms of XAP2 protein we demonstrated that mutations that disrupt XAP2-ERα interaction could no longer regulate ERα-mediated gene transcription, suggesting that XAP2-ERα interaction is crucial for XAP2 to inhibit ERα-mediated transcription; in addition, these experiments also provide evidence that the C-terminus of XAP2 protein (i.e. 2nd and 3rd TPR domains) seems to be important for mediating the XAP2-ERα protein-protein interactions.

To monitor the possible presence of XAP2 on the regulatory promoter regions of ERα target genes, we performed sequential chromatinimmunoprecipitation (Re-ChIP) assays. Results showed that XAP2 is recruited, or already present on ER-regulated promoters, together with ERα; addition of the ligand led to a lower recruitment of the ERα/XAP2 complex. Furthermore, we observed that depletion of XAP2 with siRNA led to an increased recruitment of ERα to ER-target gene promoters (**Fig. 7**).

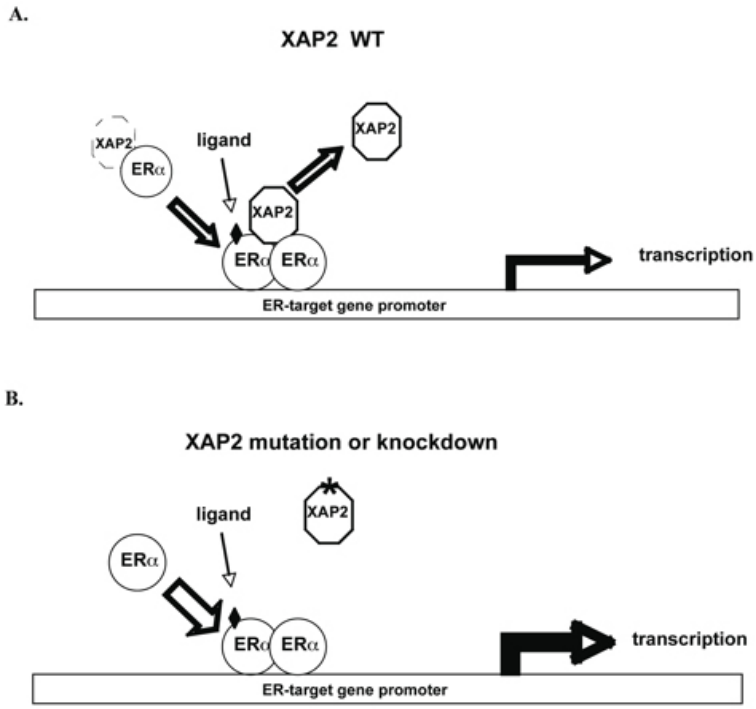

Figure 7. Schematic model of XAP2 regulation of ER-dependent transcription. **(A)** Upon the ligand (black diamond) binding, ERα is activated, WT XAP2 is starting to be released from the promoter whereas more ERα is recruited to the promoter and the target gene transcription is initiated.
(B) When XAP2 is mutated or knocked down, there is less or no functional XAP2 that could interact with ERα, ERα is recruited more actively to the target gene promoter and the transcription is highly induced.

3.3 *Paper III*: EPIGENETIC REGULATION OF GLUCOSE TRANSPORTER 4 BY ESTROGEN RECEPTOR β

Glut4 is an important regulator of cellular glucose uptake in adipose tissue and skeletal muscle. It has been shown that Glut4 activity could be modulated by ERs. However, the regulatory mechanisms behind this regulation remain unclear. In this study, we compared the roles of ERα and ERβ in Glut4 regulation and investigated the mechanism behind the regulation.

To investigate how the lack of either ER subtype affects Glut4 expression, mouse embryonic fibroblasts (MEFs) derived from WT, ERKO and βERKO mice were used. Given that Glut4 expression is restricted to skeletal muscle, heart, and adipose tissue in mice, MEFs from WT, ERKO and βERKO mice were differentiated into adipocytes in order to induce Glut4 expression. Comparison of Glut4 expressions between the ER proficient and deficient cells showed that Glut4 transcription was markedly reduced in βERKO MEF-derived adipocytes than in WT, both basally and upon induction by liver X receptor. Interestingly, there was no significant difference of Glut4 transcriptional levels in ERKO and WT MEF-derived adipocytes. These results suggest that ER regulates Glut4 expression in an ER subtype dependent manner, with only ERβ but not ERα affecting Glut4 transcription.

These changes in Glut4 expression however could not be explained by the lack of ERβ as ligand-activated transcription factor since Glut4 expression was not significantly modulated by E2 or re-introduction of ERβ. Given that Glut4 expression may be regulated by DNA methylation, we investigated if there are changes in DNA methylation of the Glut4 promoter between ERβ proficient and deficient cells. Methylation levels of the two CpG islands of Glut4 promoter were assessed by pyrosequencing of bisulfite converted DNA from the MEFs and hypermethylation of one specific CpG in island 1 (CpG11) was observed in the ERβ deficient cells. In addition, re-introduction of ERβ into ERβ deficient cells partly restored Glut4 transcription and stabilised low DNA methylation after treatment with the DNA demethylating agent 5-Aza-2'-deoxycytidine, suggesting that the presence of ERβ prevents Glut4 silencing by impeding the methylation of CpG11.

In silico analysis of the Glut4 promoter region revealed CpG11 is part of an Sp1 binding site. Our ChIP assays confirmed the binding of Sp1 to CpG island 1 in WT and ERKO MEFs. However, this binding was substantially impaired in βERKO MEFs due to the hypermethylation of CpG11. Treatment with Sp1 inhibitor

diminished Glut4 expression in WT, but not in ERβ deficient cells, suggesting that reduced recruitment of Sp1 to the Glut4 promoter is responsible for the differences in Glut4 expression.

ChIP assays showed that ERβ is the only ER subtype that is recruited to the CpG11 containing region of Glut4 promoter. Although ER has previously been shown to activate genes via Sp1 binding sites by ligand-dependent binding to Sp1, our electrophoretic mobility shift assay (EMSA) experiments suggest that the binding of Sp1 to Glut4 promoter is ERβ-independent and ERβ seems to bind to a region adjacent to the Sp1 site (**Fig 8**).

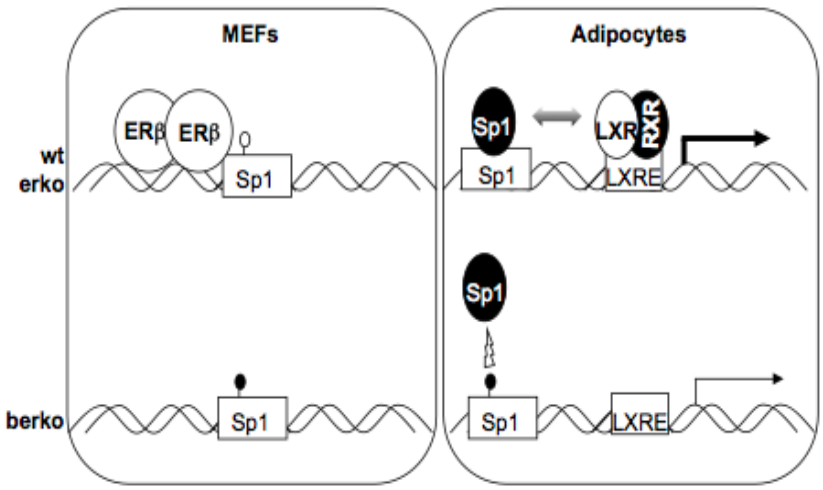

Figure 8. Model for ERβ action on the Glut4 promoter.
In WT and ERKO cells, ERβ prevents methylation of CpG11 that is part of an Sp1 binding site. In adipocytes derived from these cells, Sp1 can bind to the Glut4 promoter and activate basal transcription and inducibility by liver X receptor (LXR). In βERKO cells, on the other hand, CpG11 is methylated, which prevents Sp1 from binding to its recognition site and thus both basal and inducible Glut4 transcription are reduced. RXR: retinoid X receptor, the heterodimerisation partner of LXR.

4 DISCUSSIONS

The balance of ERα/ERβ activity is crucial in keeping normal physiological functions of estrogen. The differential effects of ERα- and ERβ-mediated estrogen action are key factors in tissue-specific response of estrogen and tightly regulated by various factors at multiple levels. The aim of this thesis is to characterize possible mechanisms and pathways by which the ER activity could be regulated in an ER subtype-specific manner.

Local protein levels of ERα and ERβ as well as ERα/ERβ ratio are important determinants for estrogen action. In ***paper I*** we demonstrate that circadian system could regulate estrogen signaling by specifically modulating the expression of ERβ. We show an example of TFs affecting intracellular levels of ER by direct regulation of ER transcription. Being controlled by different ranges of TFs, ERα and ERβ display distinct expression patterns in the body. Interestingly our studies show that ERα/ERβ ratio not only differs among different tissues and cell-types, but also varies significantly at different times of the day. In this case, in ERβ dominant tissues, estrogen responses might fluctuate between day and night whereas estrogen actions in ERα dominant tissues would show little or no diurnal variation. In tissues where both ERs are present at comparable levels, the effect of estrogen might also differ from the day to the night due to the fluctuated ERα/ERβ ratio. Daytime-dependent oscillations in estrogen action could lead to changes in susceptibility of ER-related diseases. Furthermore, this suggests that drug delivery time schedule in estrogen therapy need to be taken into account to optimize efficacy and avoid the side effect of the treatment.

Cellular factors could affect the transcription also by regulating TFs on the target gene promoter. In ***paper II***, we show that XAP2 influences ERα-mediated estrogen signaling by interacting with ERα on the promoter region of ER-target gene in breast cancer cells. Through this mechanism, XAP2 regulates the transcriptional response to estrogen in an ER subtype-specific manner, as XAP2 inhibits ERα, but not ERβ-mediated transcription. XAP2 has been suggested to have tumor suppressive function, however, the detailed mechanism is not yet clear. One possibility is that XAP2 interacts with critical proteins in tumorigenesis-related transcriptional signaling pathways. Given the important roles in tumor development, ERs and other NRs could be the candidate regulatory factors that connect XAP2 with biological outcomes. Given that the effect of XAP2 on estrogen signaling is ER subtype dependent, the disturbance of XAP2 expression,

for instance, in individuals carrying XAP2 gene mutants, would break the normal balance between ERα and ERβ actions by over-activating ERα mediated estrogen signaling pathway. As a consequence, these individuals could have higher risk of developing estrogen related tumors since only the "proliferation promoter" ERα, but not the "proliferation inhibitor" ERβ is stimulated. This finding suggests a possible mechanism involved in the tumor suppressor function of XAP2. However, further investigations, especially clinical studies, are necessary to substantiate the suppressive role of XAP2 on tumor development.

In ***paper III*** we identify a novel epigenetic mechanism under the ER subtype-specific regulation of gene expression. We show that ERβ, but not ERα, is essential in maintaining the unmethylated state of CpG11 in Sp1 binding site of Glut4 promoter, which is required for normal Sp1 recruitment and Glut4 transcription in adipocytes. ERβ but not ERα is highly expressed after egg fertilization when global demethylation and remethylation, suggesting that ERβ is the only ER subtype involved in establishing the methylation pattern of genes such as Glut4. Furthermore, exposure to endocrine disruptive chemicals such as bisphenol A or diethylstilbestrol during sensitive developmental stages is known to "prime" the exposed organism to different metabolic disorders such as obesity and diabetes type 2. This is possibly due to the disturbance of normal ER activities since ERs are important regulators in fat and glucose metabolism. Therefore, the mechanism revealed in this paper provides a possible link between changes in glucose tolerance and epigenetic alterations observed after exposure to endocrine disruptive chemicals in early development. However, the ER regulation of Glut4 seems to be complex and might involve different mechanisms and pathways, further investigations are required to elucidate the whole picture of the regulation.

5 CONCLUSIONS

ER subtype specific activities are involved in many physiological and pathological processes. Understanding the mechanisms of ER subtype specific regulation is important in studying the estrogen signaling. In this thesis, we show how the activities of ERα and ERβ being modulated by various cellular factors at different levels. We have identified three novel pathways in mediating the ER subtype specific regulatory effects, which are summarized and presented in **Fig 9**.

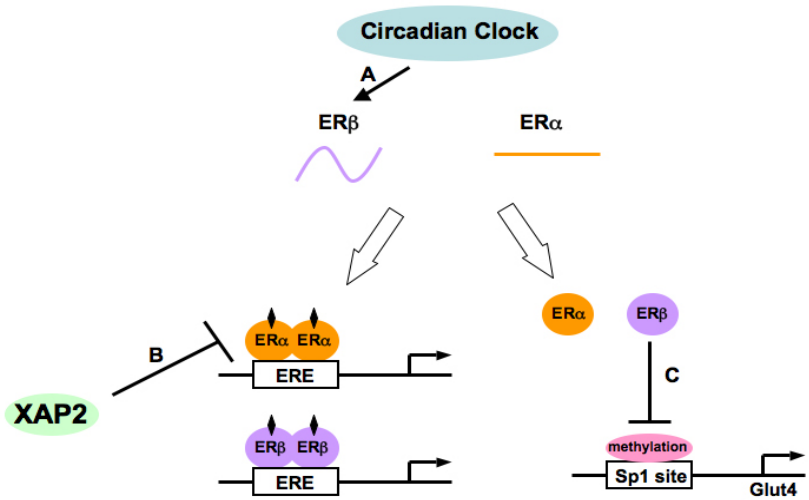

Figure 9. The three pathways of mediating the ER subtype specific regulatory effects presented in this thesis. **(A)** The expression of ERβ is regulated by components of the molecular clock (***paper I***); **(B)** XAP2 inhibits ERα-mediated estrogen signaling (***paper II***); **(C)** Epigenetic regulation of Glut4 by ERβ (***paper III***).

6 FUTURE PERSPECTIVES

The meticulous and thorough regulation of ERα and ERβ activation is essential in maintaining the normal biological function of estrogens. This regulatory process involves the crosstalk of various mechanisms and pathways. Therefore, getting a systematic view of the regulation is important. In this case, high throughput screening such as RNA-seq, ChIP-seq, RNAi screening as well as epigenome-wide association studies (EWAS) need to be applied to better understand the interaction network of different pathways. Furthermore, clinical studies, where ERα/ERβ activities are determined in patients suffering estrogen-related diseases, will provide valuable information for understanding the biological relevance of the regulation.

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