§1. INTRODUCTION

Estrogen receptor-related receptors in the role of estrogen related cancer and the nuclear signal pathway

1.1 Research background

Estrogens have important physiological functions in the proper development, sexual differentiation and maintenance of both the female and male reproductive system. Abnormal estrogen activity has been implicated in numerous diseases, including osteoporosis, coronary heart disease as well as some estrogen related malignancies such as breast cancer, endometrial carcinoma and ovarian cancer [1]. The pleiotropic effects of estrogen are mediated by two classical estrogen receptors (ERs) known as ERα and ERβ (according to the American Nuclear Receptors Nomenclature Committee 1999, named as NR3A1 and NR3A2) [2], which are ligand-activated transcription factors [2, 3]. As members of the nuclear receptors (NRs), ERs regulate the target gene expression via recruiting co-regulators to a specific DNA element, the estrogen responsive element (ERE) [4-6]. However, not all the estrogen responsive effects can be explained by this classical estrogen-ER-ERE signal transduction theory. There might be some bypass to the classical estrogen pathway or co-regulated mechanism in the estrogen-ER signal pathway.

In recent years, some interesting results were reported concerning to the orphan nuclear receptors, which also belong to the NRs super-family. In contrast to the classical ligand dependent definition, these nuclear receptors could be activated by no known ligand on a constitutive manner and be named as “orphan receptors” [7-11]. Most orphan receptors were identified by two-yeast-hybridization screening with cDNA libraries using the conservative DNA binding domain of nuclear receptors [8-10]. Although these “orphan” receptors shared a high conservative gene and protein structure with their homological receptors, their functions were found different from the classical receptors [9-11]. There is also a subfamily of orphan nuclear receptors closely related to the ERs named estrogen receptor-related receptors (ERRs) [9,11,12]. Due to their high homology with ERs, the members of ERR family likely transduce signal via cross-talking with other nuclear receptors through common ER binding sites as well as ERR specific binding sites [8-12]. The target genes may include not only the classical Estrogen-ER complexes responsive genes but also some ERRs specific genes. An ongoing research confirms that various orphan nuclear receptors play an important role in the pathway of nuclear

signal transduction and regulation of the genes transcription [13-15]. Indeed, one of the remaining questions concerning orphan receptors is how their transcriptional activities are regulated, whether they have natural ligand remains to be identified as classical receptors or do they rather have a constitutive activity in a ligand-independent manner?

1.2 ERRs encoding gene

1.2.1 The chromosome position of ERRs encoding gene

Up to now, the ERR subfamily is known to comprise three members ERRα, ERRβ and ERRγ (Nuclear Receptors Nomenclature Committee 1999, named as NR3B1, NR3B2 and NR3B3, respectively), which also belong to the group III of the NRs super-family with ERs, glucocorticoid receptor (GR, NR3C1), mineralocorticoid receptor (MR, NR3C2), progesterone receptor (PR, NR3C3) and androgen receptor (AR, NR3C4) [9-12,16]. The ERRα and ERRβ were the first orphan nuclear receptors identified by low-stringency screening in the kidney cDNA libraries with a probe corresponding to DNA binding domain of human ERα, and were thus appropriately referred to as estrogen receptor-related receptors [9]. ERRγ, the third member of ERR subfamily, was identified 10 years later by two-yeast-hybridization screening with the glucocorticoid receptor-interacting protein 1(GRIP 1) as a bait [12]. Each member of ERRs has itself different isoforms [9,16,18,21]. A major isoform of ERRα, ERRα-1 was isolated from the human endometrial carcinoma cell line RI-95-2 based on its binding to the steroid factor-1 (SF-1) responsive element (SFRE) [18, 21]. After transcription and translation, the human ERRα-1 gene encodes a 422 amino-protein (42kDa), which is smaller than ERRα protein (512 amino-protein, 55.3 kDa) [18]. Using fluorescent in situ hybridization, encoding ERRα gene was mapped onto the chromosome 11q12-q13 and ERRβ was mapped onto the chromosome 14q24.3 [18,22,23]. The ERRγ was also identified on the chromosomal 1q41 [13].The gene maps of ERRs family and the different transcripts of ERR isoforms can be seen in Figure 1.1 (A-C). Analysis on the genomic organization and promoter characterization shows that the nucleotic sequence adjacent to the transcription start sites of human ERR (ESRL1) lacks the typical TATA and CAAT boxes, but it is GC rich and contains 10 consensus Sp1-binding elements and two E boxes [18]. The human genome also encodes an ERR-related pseudogene, which is located on the chromosome 13q21. It was the first reported as a pseudogene associated with a member of orphan nuclear receptor family [23]. All the members of ERRs display a high degree of sequence homology with their DNA binding domain (DBD) and ligand binding

domain (LBD) [11, 12, 16]. Moreover, the ER family also has a high degree sequence homology with ERR family (see the Figure 1.2), which strongly indicated that these two families probably bind to the same element on the target gene promoter and may have some overlap functions.

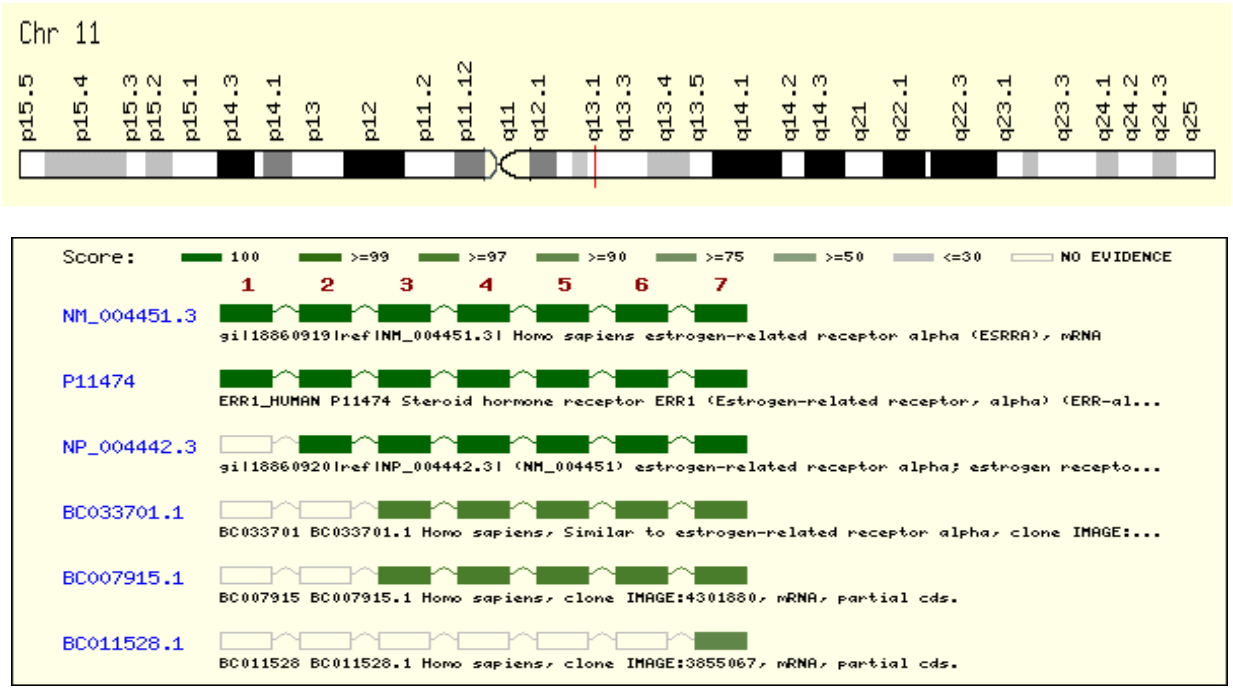

Figure 1.1 A:

The position of ERRα encoding gene and the different transcriptions of ERRα mRNA

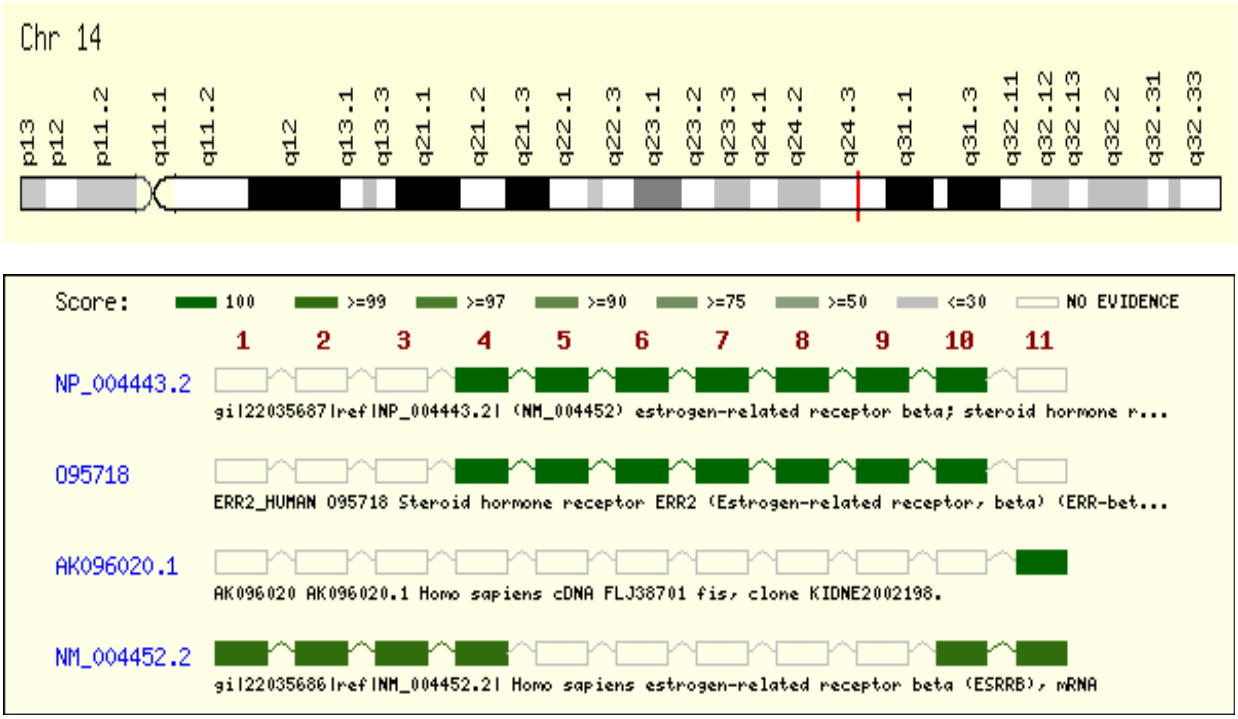

Figure 1.1.B:

The position of ERRβ encoding gene and the different transcriptions of ERRβ mRNA

Figure 1.1.C:

The position of ERRγ encoding gene and the different transcriptions of ERRγ mRNA

1.2.2 Structure of ERR encoding genes

Similar to the conventional nuclear receptors, ERRs are organized into several modular regions, which have distinct biological functions (see Figure 1.3) [24-26]. The A/B regions are located at the N-terminus and include the activation function 1 (AF-1 region). The C region includes the DNA binding domain (DBD), which is the most conserved through evolution and contributes to the special DNA-binding by two zing-finger motifs. Cooperating with the zing-finger motifs, the P-BOX in the DBD of nuclear receptor can sequence-specifically recognize the responsive elements in the promoter of target genes [27]. Furthermore, according to the different binding models between the nuclear receptor DBD and the responsive element of target gene, the nuclear receptors can be organized into different heterodimers or homodimers [27]. The D region is considered as a hinge region, bridging contact with the N-terminal and C-terminal. The E region contains the assumptive ligand-binding domain (LBD), which contains the ligand binding hydrophobic pocket and contributes to receptor dimerization [24-27]. In ERs, embedded within the LBD is a hormone-dependent transcription activation region (AF-2 region) [27-28]. In some studies, the AF-2 was considered as a major function domain for binding with cooperators [29]. However, the functions of D domain and F domain are still poorly understood [28].

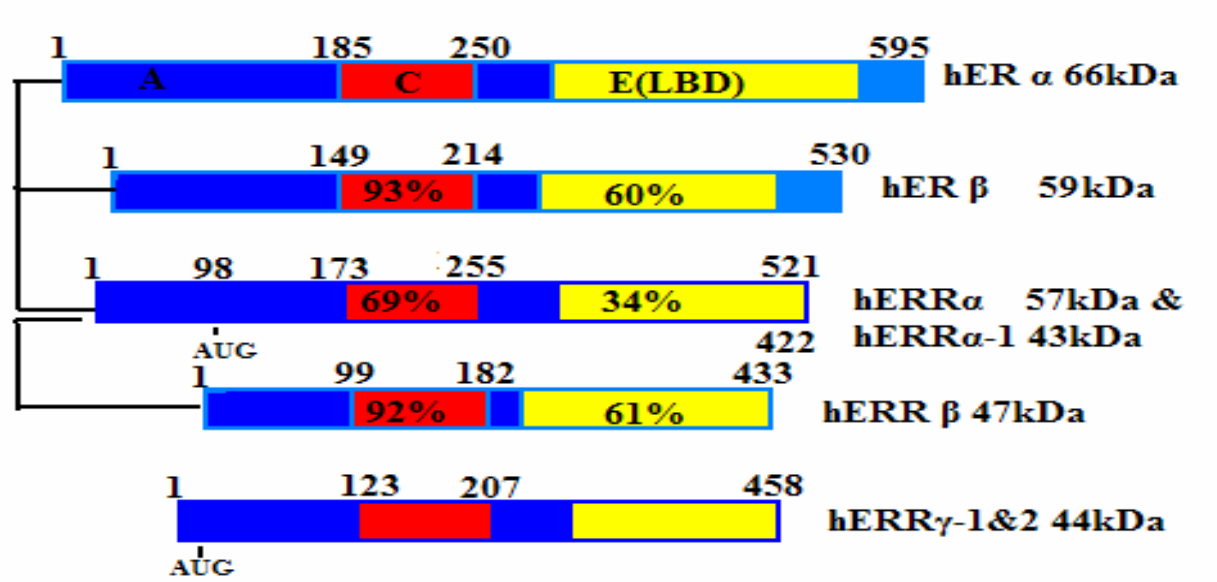

Figure 1.2: The structure homology between the ER family and ERR family. The percentage on the C domain (DBD) and E/F domain (LBD) showed the different levels of similarity between subtypes of these two families. ERα and ERβ have 93% and 60% identity in the DBD and LBD, respectively; ERα and ERRα have 69% (70% with ERRα-1) and 34% (35% with ERRα-1) identity, respectively; ERRα and ERRβ have 92% and 61% identity, respectively.

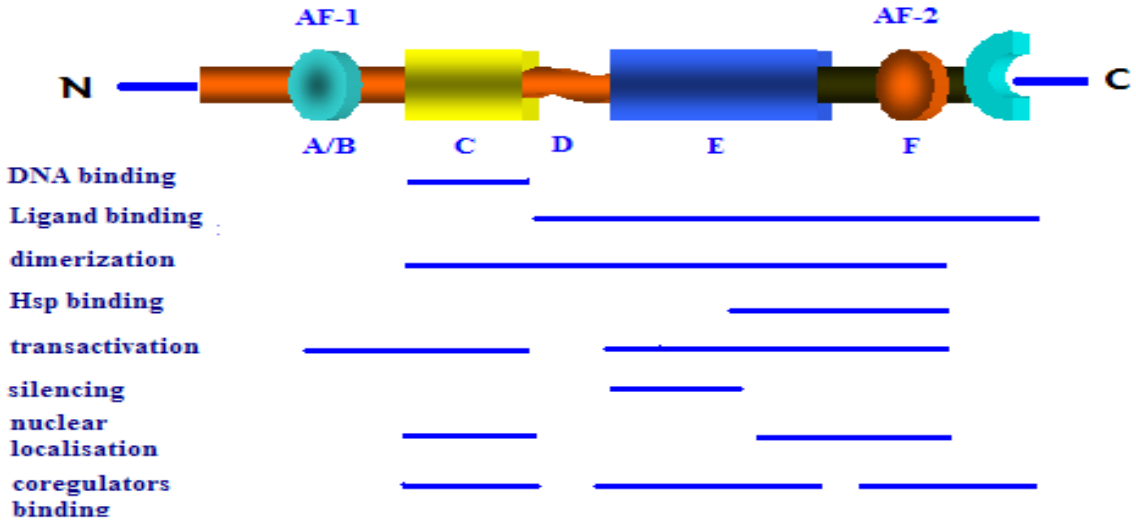

Figure 1.3: Scheme of human ER and ERR families' structure and their function. On ER, there is an F domain in the C terminal, whose function is poorly understood. And AF-2a transcript action domain is only for ER. The AF-2 domain is very important for ER to exert its function.

Sequence analysis comparing all of the NR3 family has shown that the ERs and the ERRs form one branch of group III nuclear receptors, which recognize the hormone responsive element (HRE) sequence as 5`-**AGGTCA**-3`, whereas the four other steroid

receptors recognize the sequence as 5`-**AGAACA**-3` and form another branch of group III nuclear receptors [21,28,29]. The genome of *Drosophila melanogaster* encodes a single member of this group, an ERR ortholog [22], indicating that all group III nuclear receptors might have originated from the same ancestral ERR. ERRs display the same domain organization as do classical receptors and keep conservation through evolution, which strongly suggests these receptors may play critical roles in estrogen signal pathway.

1.3 Expression pattern of ERR

ERRα appears to be widely distributed, both in the developing embryo and adult tissues [33-34], although it is the most abundant in the uterus (cell lines), prostate, brain [11], heart, skeletal muscle [18] and brown adipose tissue [30]. During the fetal mouse development, ERRα mRNA can be detected in the embryonic stem (ES) cells and the development of heart and skeletal muscle, the central and peripheral nervous system, the epithelium of the intestine and urogenital tract [33]. This expression begins at the time of chorioallantois fusion in the placenta and through the developing of heart, intestine, brain, spinal cord, brown fat and bone [28,34]. It suggests a role of prime importance for this receptor throughout a lifespan. Nevertheless, the mechanism of the function of ERRα is still not correctly understood. In contrast to the widespread expression pattern of ERRα and ERRγ, ERRβ is present early in the extra-embryonic ectoderm during the development of the placenta. The ERRβ is also found in a very low concentration in a few specific rat tissues (kidney, heart, hypothalamus, hippocampus cerebellum, rat prostate, specific areas of the mouse brain) [11,28,35,36]. However, in mice lacking ERRβ, trophoblast stem cell differentiation is impaired and the placenta fails to develop normally [35,36]. Thus, ERRβ is essential for reproduction and normal development. Similar to ERRα, human ERRγ transcripts can be detected widely in brain, lung, bone marrow, adrenal and thyroid glands, trachea and spinal cord, with very high levels in the fetal brain, and at lower levels in the kidney, lung and liver [12,13,28,37,38]. By the Northern-Blot analysis, expression of ERRγ could be detected as early as in the E12.5 day of mouse embryo period [38]. In summary, the expression of ERR are widely detected in the fetal and adult tissues. In contrast to the broad distribution, the knowledge about their function is still very limited.

1.4 Function of ERRs and intracellular interaction with ERs

Although the ERRs were discovered more than 10 years ago, the knowledge about the

biology and function of ERRs is still very limited. Recent results obtained from the research on ERRs have shown that the ERRs share target genes, co-regulatory proteins and DNA binding sites of action with the ERs [19,39-42]. Despite the high degree of sequence similarity with ERs in the construction, the ERRs do not bind to the natural estrogen such as E_2 [43,44]. A triple mutant in the P-BOX motif of DBD may be one reason for different binding characters between ER family and ERR family with the natural estrogen. The identification of naturally occurring ligands for ERRs family members has remained elusive. However, in silico superimposition of the ligand-binding pocket of ERRα and that of ERα has revealed a greater level of local alanine residues identity, suggesting that structurally close ligands could probably be bound by both receptors [43,45,46]. There are also publications showing that the ERRα possibly has a natural ligand yet to be identified. The activity of ERRα-1 has reported to be antagonized by two organochlorine pesticides and toxaphene [44,47]; the activity of ERRα-1 was also reported to be depend on an unidentified serum component [43]. Diethylstilbestrol (DES) appeared to inactivate all three members and may be the ligand of ERRβ [48], while 4-hydroxytamoxifen (4-OHT) seemed to bind only to ERRβ and ERRγ and selectively inactivate ERRγ in cell based assay [49,50]. Recently, PPARγ coactivator 1β was reported as an ERR ligand and contributed to the control of energy balance in an *in vitro* experiment [51]. Strikingly, all these compounds are more or less connected to estrogen signaling and only showed repression on ERR's transcriptional activities. They can be thus considered as antagonists (if ERRs possess natural ligands) or inverse agonists (if ERR's transcriptional activities are truly constitutive) [12,28,39].

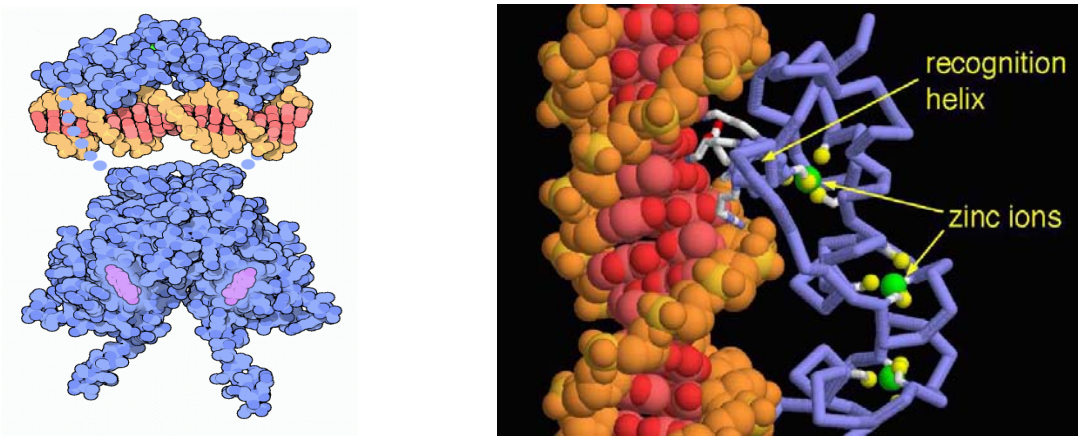

Figure 1.4: The binding model of ERRs and target gene. By the zing-finger motif on the DBD domain, the ERRs recognized and bind to the ERRE element on the target genes and drive the transcriptions.

ERR monomers preferentially recognize the consensus extended half-site 5`-**TnA-AGGTCA**-3` (see the Figure 1.4) with a high-affinity, referred to as the ERR-responsive element (ERRE) [19,21,23,52]. This class of binding site is also recognized by the monomeric orphan nuclear receptor steroidogenic factor-1 (SF-1; NR5A1), a regulator of the steroid biosynthesis pathway that is also essential for the development of the hypothalamic–hypophiseal–adrenocortical axis [32]. Specifically, the members of ERR family can recognize variants of ERE including the perfect ERE (estrogen receptor responsive element), ERRE (estrogen receptor related-receptor responsive element), perfect SFRE (steroid factor-1 receptor responsive element), a perfect SFRE and an imperfect ERE [28,46] and TREpal (palindromic thyroid hormone responsive element) [53] by dimers or monomers. Though the activation mechanism of ERR remains totally unknown, the ERR need to recruit the co-regulated protein to perform their function [54,55]. ERRs were observed to compete with ER to bind to the steroid receptor coactivator (SRC) family, which is essential for ER mediated gene transcription, the integration of intracellular signaling pathways and control of the cell cycle [28,54-56]. On the other hand, in absence of exogenous ligands, all the ERRs, had

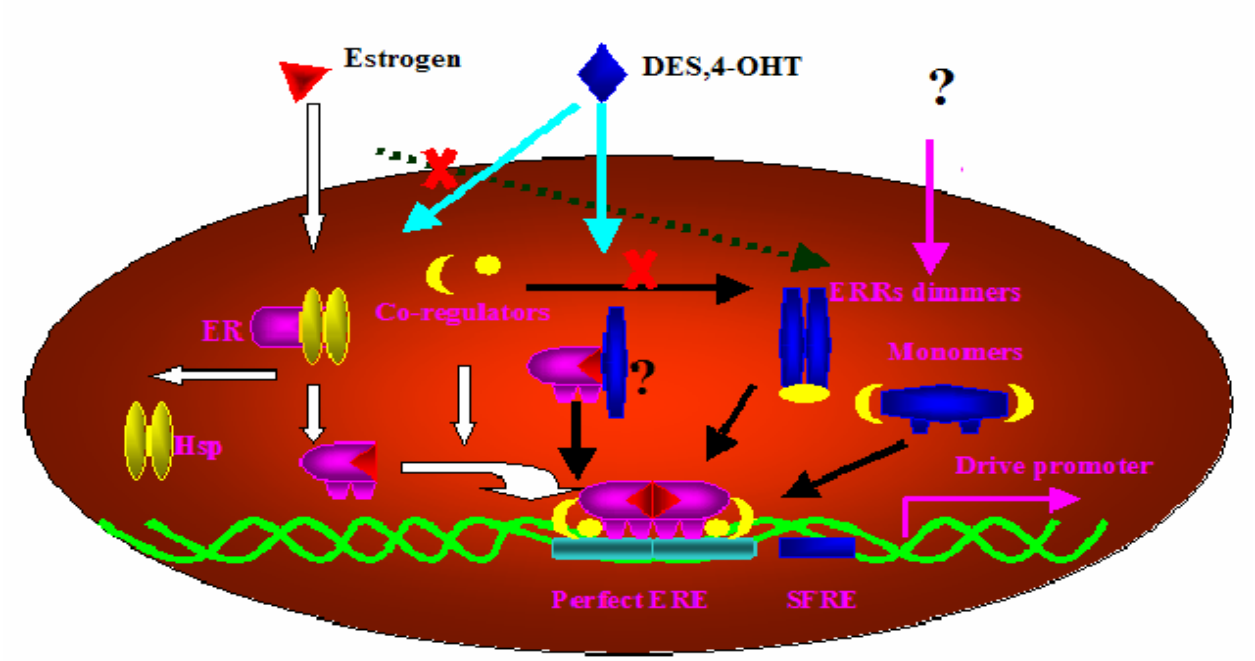

Figure 1.5: The intercellular interaction between ERs and ERRs. The classical E-ER signaling: ER is activated by natural estrogen or synthesis estrogen such as DES and 4-OHT, then the E-ER complex changes its structure to recognize and trigger the target gene expression following binding to ERE with co-regulators (white arrows) . ERR can reorganize not only the ERE, but also ERRE and SFRE on a constitutive manner.

been shown to interact with some coactivators and activate target genes transcription in a constitutive manner [12,21,31,39,40,57]. Thus, ERR may play an important role in the response of some genes responsive to estrogen via heterodimerization with ERs or directly competition with ERs for binding to ERE or coactivators [16,41,58]. ERRα-1 can work as a modulator of estrogen responsiveness as an estrogen-independent activator by function of either an active repressor or a constitutive activator of ERE-dependent transcription. The ERRs can actively influence on the estrogenic responsive gene function, suggesting that there is a key way for ERRs to regulate the pathway of estrogen–ER signal pathway, though the mechanism of this cross-talking between these two receptor subfamilies is still not clear. These observations offer additional layers of regulatory complexity for intracellular interaction between ERRs and ER (the probable mechanisms were summarized in the Figure 1.5). The increasing knowledge about ERRs function will promote the understanding of the complex nuclear receptor signaling transduction [54].

In context with other transcription factors and co-regulators, the ERRs regulated the target gene activity in a ligand-, cell type-, responsive element-, and promoter context-specific manner. ERR play an active role in bone morphogenesis such as regulation of the osteopontin gene, bone resorption, and osteoprogenitor cell proliferation and differentiation [31,34,59]. hERRα regulates the transcriptional activity of human lactoferrin gene [19,58], human pS-2 gene [40], human medium-chin acyl coenzyme A dehydrogenase gene [23,61,62], thyroid receptor α gene [32], aromatase gene [63], osteopontin gene [31,34,64] and small heterodimer partner (SHP) orphan nuclear receptor [57]. The major isoform of the human ERR alpha gene, hERRα-1, can sequence-specifically bind to a consensus palindromic estrogen responsive element (ERE) and directly compete for binding with estrogen receptor alpha (ERα) [40,42]. ERRα-1 activates or represses ERE-regulated transcription in a cell type-dependent manner, repressing the ERE mediated transcriptions in ER-positive MCF-7 cells while activating the ERE mediated transcriptions in ER-negative Hela cells [41, 42]. The hERRβ function as a potent cell-specific, receptor-specific repressor of transcriptional activity mediated by glucocorticoid receptor [65]. The expression of two isforms of ERRγ, mERRγ-$_1$ and mERRγ-$_2$ in the mouse central nervous system during the embryonic development and the brain of adult mice suggests that they may take part in the differentiation and function of the brain [37,38,66]. Though in the past years numerous interconnections between the ERR and Estrogen signaling have been documented and

are thoroughly discussed in recently published reviews [7,28,46,66], little is known about the *in vivo* function of the ERR. It still needs more working to focus on.

1.5 ERRs in the Estrogen Related Cancer

Breast, uterus and ovary were considered as the classic estrogen target organs, ERs are highly expressed in these tissues and their malignancies. Therefore, the cellular malignant mechanisms of these tissues were rate-limited with estrogen-ER signal pathways. In estrogen-related cancer such as breast, endometrial and ovarian carcinoma, ERα mediates estrogen responsive cell proliferation and plays a crucial role in the etiology of the cancers [67-69]. Especially in the breast and endometrial carcinomas, ERα has been established as the single most important genetic biomarker and target for cancer therapy. Selection of the patients with ER-positive expression increases endocrine therapy responsive rates based on the anti-estrogen [70]. However, not all anti-estrogen therapies have an affect on the ER positive patients. On the other side, the status of some patients without ER expression can be improved by the combination of anti-estrogen and progesterone agnonist treatment. This suggests some other net-regulations happen in the estrogen nuclear signal pathways.

ERRα was detected in the breast carcinoma (cells and tissue), endometrial carcinoma (cells and mouse uterus), and mouse ovarian tissue [9,41,42,70,71]. Research on the *in vitro* established mammary carcinoma cells and cervical cancer showed that both ERα and ERRα-1 can bind to palindromic ERE sequence, and there is a competition between these two nuclear receptors [41,44]. ERRα repressed ER-mediated gene transcription in the ERα-positive MCF-7 cell, but in an ERα-negative CV-1 cell, the ERRα activated the same gene transcription [42]. Abnormal estrogen synthesis was considered as a major inducing factor to the development of estrogen-related cancer. To adjust the estrogen synthesis, aromatase activity was the most important limiting step. Promoter I.3 and II are thought to be important promoters driving abnormal aromatase expression/estrogen synthesis in breast tumors. A regulatory element (S1) behaves as an enhancer or repressor between these two promoters. ERα can bind to the S1 element and repressed the aromatase promoter activity as a feed-back mechanism to suppress abnormal over estrogen biosynthesis [72]. In contrast to ERα, ERRα was also found to be the major protein interacting with the silence element (S1) of the human aromatase gene in the breast tissue as an enhancer. Its ability to interact with ERα and to modulate aromatase expression/estrogen biosynthesis suggests that ERRα plays a critical role in the normal

breast development and important in the pathogenesis and maintenance of breast cancer [63]. The importance of ERRs in human breast cancer was also assessed by comparing their mRNA profiles with established clinicopahtological indicators and mRNA profiles of ERs and ErbB family members [70,71]. Moreover, ERRα and ERRγ are candidate targets for new therapeutic development.

It was confirmed that the pS2 gene, a human breast cancer prognostic marker, which promoter has an ERE, can be regulated by the ERRs as a target gene [40]. Consensus ERE were reported in several target genes such as: lactoferrin, pS2, c-fos, c-jun, c-myc and EGF receptor, epidermal growth factor, cyclin D1, breast cancer-1 (BRCA-1) gene [55,66,67]. Those genes were rated-limiting with the cancer development. It means that all these genes are potential target genes regulated by the ERRs based on their ability of binding to variants ERE and ERRE. So, the ERRs may play an important role in the etiology of some estrogen related cancers such as breast cancer, endometrial carcinoma and ovarian cancer.

1.6 Research Objectives of ERRs

This review underlines that ERRs represent to be important pleiotropic modulators of estrogen-ER mediated target genes and ERR specific genes transcription. Their contribution to the estrogen signaling is indubitable and therefore the search for potential ligands and new target genes should be primary importance in future studies. Major goals of future studies will be to identify ER and ERR target genes using technologies such as chromatin immunoprecipitation assays and gene arrays, and to confirm the regulation of these genes by each receptor subtype in both cell and animal-based models with the help of pharmacological and genetic tools. These objectives may include:

- Identification of the target genes regulated by the ERR family
- Functions of ERR in estrogen-induced cancer
- Evaluation of ERR as a therapeutic target, especially on the estrogen-independent tumors.
- Relationship between ERR and SERM (select estrogen receptor modulator)

1.7 The study significance of the expression of ERRs in the ovarian cancers

Epithelial ovarian cancer is the leading cause of death from gynecological malignancies in western countries [73]. One of the reasons for the poor prognosis is the high rate of

advanced tumors at the time of diagnosis: about 75% of all patients are diagnosed in FIGO-stage III of IV [74]. High serum level of estrogen has been implicated as a risk factor of ovarian carcinoma, but the cellular signal pathways involved are not completely clear so far [2,3,68]. Moreover, ERα and ERβ are found highly expressed in normal human ovaries, benign ovarian tumors, borderline and malignant ovarian tumors, as well as in primary cultures of normal human ovarian surface epithelial (HOSE) cells and established ovarian cancer cell lines [68,75,76]. However, the clinical role of ER in ovarian cancer is not as important as in breast cancer, although the ovary is the main source of estrogen in women [75]. Furthermore, expression of ER mRNAs have not enough prognosis value in the hormone-related ovarian cancer [75,76]. There remains a great need to develop the role of steroid hormones and the nuclear signal pathway in the carcinogenesis of ovarian cancer.

In contrast to the ligand-dependent classical ER, ERRs were found to activate in a constitutive manner without any exogenous estrogen stimulation. ERRs can activate some estrogen responsive genes such as pS2 and aromatase genes in breast cancer cell lines [40-44] and serve as a biomarker independent of the estrogen-ER signal pathway [12,28]. It has been suggested that there is a key role of ERRs to regulate the estrogen signal pathway in tumors, though the mechanisms of this crosstalk are still unclear [11,28,71,72]. Thus, the ERR family may be the potential biomarkers involved in the ovarian cancer. The studies on ERRs in the ovarian cancer may improve the knowledge about the complex molecular mechanism of the nuclear signal transduction and regulation. However, there the role of ERR family in ovarian cancer are not clearly understood yet.

Do ERRs express in the ovarian cancer? If they are expressed, do ERRs play a critical role in the etiology of ovarian cancers? Moreover, what kind of *in vivo* associations are between the members of ERR family and ER family in the ovarian cancer? In this study, is there a potential utility of ERRs as novel ovarian biomarkers? To determine whether this subfamily of orphan nuclear receptors might be associated with ovarian cancer I studied the expression of the major isoforms of the ERRs family, including hERRα, hERRβ and hERRγ, in ovarian cancer cell lines as well as in malignant and normal ovaries by LightCycler quantitative RT-PCR and immunoreactivity. Furthermore, I also investigated the clinicopathological relevance of these orphan receptors.